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(57) Abstract

It has been discovered that BRCA1 mediates transcriptional activation of *p21* and other tumor suppressor genes in a p53-dependent and p53-independent manner. Assays and compositions for identifying compounds that enhance or repress cellular proliferation *via* these newly-discovered BRCA1-mediated pathways are disclosed. Diagnostic methods and methods of controlling cell proliferation by altering the pathways are also disclosed.

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COMPOSITIONS AND METHODS FOR CONTROLLING BRCA1-MEDIATED P53-DEPENDENT AND -INDEPENDENT REGULATION OF TRANSCRIPTION

This application claims priority to U.S. Provisional Application Serial No. 60/080,146, filed March 31, 1998, incorporated by reference herein.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Institutes of Health, Grant No. CA57601.

FIELD OF THE INVENTION

This invention relates to regulation of cell proliferation. In particular, this invention provides plasmids, cell lines, kits and assay methods for identifying compounds that affect p53-dependent and p53-independent regulation of transcription by BRCA1, as well as diagnostic and therapeutic methods relating thereto.

BACKGROUND OF THE INVENTION

Various scientific and scholarly articles are referred to in parentheses throughout the specification. These articles are incorporated by reference to describe the state of the art to which this invention pertains.

Breast cancer is one of the most common malignancies affecting women. About 5-10% of all cases are estimated to be familial. Susceptibility To early-onset breast and ovarian cancer is conferred by mutations in a gene on chromosome 17q21. This gene termed *BRCA1*, has been identified by positional cloning (Miki et al., 1994, Science 266:66-71). Mutations in the *BRCA1* gene account for about 50% of inherited breast

cancer cases and 80% of families predisposed to both breast and ovarian cancer.

Much effort has been recently directed at understanding the biochemical function of *BRCA1*. The human *BRCA1* gene encodes an 1863 amino acid nuclear protein that is expressed in a wide variety of adult human tissues (Marquis et al., 1995, Nat. Genet. 11:17-26). Evidence implicates a role for *BRCA1* in the control of gene expression. *BRCA1* contains a nuclear localization signal (Thakur et al., 1997, Mol. Cell. Biol. 17:444-452), a C-terminal domain that transactivates gene expression when fused to a heterologous DNA binding domain (Chapman and Verma, 1996, Nature 382:678-679; Monteiro et al., 1996, PNAS 93:13595-13599) and BRCA1 has been found as a component of RNA polymerase II (Scully et al., 1997, PNAS 94:5606-5610).

Several lines of evidence suggest that BRCA1 protein may play an important role as a suppressor of cell proliferation. Antisense oligonucleotides to *BRCA1* mRNA accelerated the growth of normal and malignant mammary epithelial cells (Thompson et al., 1996, Nat. Genet. 9:444-450). Similarly, NIH 3T3 cells with a stable expression of antisense *BRCA1* mRNA showed accelerated growth rate, anchorage independent growth, and tumorogenicity in nude mice unlike the parental cell line and stable cell lines expressing sense *BRCA1* mRNA (Rao et al., 1996, Oncogene 12:523-537). In addition, transfection of the wild-type *BRCA1* gene inhibited the growth of breast and ovarian cancer cell lines (Holt et al., 1996, Nat. Genet. 12:298-302). Retroviral introduction of wild-type *BRCA1* gene into the already established MCF7 human breast tumors inhibited the growth of the tumors significantly and also increased the

survival of mice (Holt et al., 1996, *supra*). Another evidence that *BRCA1* may have an antiproliferative function is that human breast cancers involving mutations in *BRCA1* feature increased proliferation, aneuploidy, high S-phase fraction and high mitotic grade (Marcus et al., 1997, Cancer 80:543-556).

Evidence also suggests that BRCA1 may activate cell proliferation. The protein p21 is a universal cell-cycle inhibitor that specifically binds cyclin-CDK complexes and proliferating cell nuclear antigen, thereby serving as a potent inhibitor and effector of cell-cycle checkpoints (Sherr and Roberts, 1995, Genes Dev. 9:1149-1163). Cells from BRCA1-null mouse embryos have increased levels of p21 mRNA, which suggests that BRCA1 may suppress p21 expression during development to allow cell growth (Hakem et al., 1996, Cell 85:1009-1023).

A number of observations have also linked *BRCA1* to DNA damage response pathways (Brugarolas and Jacks, 1997, Nature Med. 7:721-722). *BRCA1* normally co-localizes with Rad51, the human homolog of the *Escherichia coli* RecA protein, at nuclear dot structures that may be sites of checkpoint processing in S-phase cells (Scully et al., 1997, Cell 88:265-275; Tashiro et al., 1996, Oncogene 12:2165-2170). Following exposure to DNA damaging agents, BRCA1 becomes hyperphosphorylated and disperses from dot structures and then dynamically accumulates at PCNA-containing replication structures, suggesting a role in the checkpoint response (Scully et al., 1997, Cell 90:425-435).

It is not known how BRCA1 affects gene transcription in normal cells and how mutations in BRCA1 cause cells to become cancerous. Lack of this information hinders the development of methods of

treatment of mutant BRCA1-induced tumors. In particular, it is very desirable to have a model system of BRCA1 activation of gene transcription so that alternate ways of activating the tumor-suppressing genes can be devised for tumor cells.

SUMMARY OF THE INVENTION

In accordance with it the present invention, two ways in which BRCA1 inhibits cell proliferation have been discovered. First, BRCA1 inhibits S-phase cell cycle progression by transactivating expression of the CDK-inhibitor $p21^{WAF1/CIP1}$ in a p53-independent manner. Second, BRCA1 interacts with p53 *in vitro* and *in vivo*, and increases *p53*-dependent transcription from the *p21* and *bax* promoters. These pathways of BRCA1 action provide novel targets to which pharmaceutical agents capable of affecting cell proliferation and apoptosis can be isolated, and can then be used in the treatment and control of cellular proliferation disorders.

One aspect of the present invention is drawn to compositions and methods for use in assay systems to identify such novel pharmaceutical agents. One method uses an *in vitro* or cell-based system that measures the ability of a test compound to enhance or interfere with the association between BRCA1 and p53. This method comprises allowing BRCA1 and p53 to associate in the presence or absence of the test agent, separating the BRCA1/p53 complexes, and determining the amount of complex formed in the presence, as compared to the absence, of the test compound.

Another method determines the effect of the pharmacological agent on p53-dependent and -independent BRCA1-mediated gene transcription. This method comprises

the culturing of various cell lines containing reporter gene(s) driven by the *p21* or *bax* promoters with and without the agent and assaying the activity of the reporter genes. Using appropriate cell types, e.g.,BRCA1-/BRCA1- and WT cell lines, this method can be used to determine the ability of a pharmacological agent to coactivate p53 in a manner similar to BRCA1 coactivation of p53.

A variation of the above-described method determines the effect of a pharmacological agent on p53-dependent and p53-independent BRCA1 activated apoptosis or cell cycle arrest. In a preferred embodiment, this method comprises the culturing of $p53^{+}/p53^{+}$ and $p53^{-}/p53^{-}$ cell lines with and without the agent and assaying for apoptosis or cell cycle arrest, using standard methods.

According to another aspect of the present invention, isolated nucleic acids and/or polypeptides are provided, corresponding to (1) the BRCA1/p53 protein binding domains; and (2) the *p21* transcriptional regulatory region required for BRCA1-mediated, p53-independent transactivation, which have been identified in accordance with the present invention. In a preferred embodiment, the nucleic acid molecule encodes, or the polypeptide comprises, residues 224-500 of the BRCA1 protein. In another preferred embodiment, the nucleic acid molecule encodes, or the polypeptide comprises, residues 300-395 of the p53 polypeptide. In another preferred embodiment, the portion of the *p21* promoter comprising the transcription start site and 5' sequence to -143, specifically the region 93 to 143 nucleotides upstream of the transcription start site, is provided.

The aforementioned isolated nucleic acids or peptides are useful for some of the assays described

above, as well as for diagnostic assays to determine the predisposition of an individual to developing certain tumors, based on the presence or absence of functional mutations that disrupt BRCA1-mediated control of cell proliferation through *p21*, *bax* or other downstream targets, either dependent on or independent of p53.

According to other aspects of the invention, methods are provided to control cell proliferation, by up-regulating or down-regulating p53 dependent or independent BRCA1-mediated transcriptional activation of *p21* or other downstream targets involved in apoptosis or cell cycle arrest. Up-regulation of these pathways should result in suppression of cell proliferation either by inducing cell cycle arrest or apoptosis, thereby providing a useful cancer therapy. Down-regulation of these pathways should result in increased cell proliferation, which may be useful in cell culture for expansion of recalcitrant cell lines, or which may be useful as a research tool.

In another aspect of the present invention, kits are provided for practicing the assays and diagnostic methods set forth above. These kits contain one or more plasmids, cell lines, antibodies, reagents and the like, along with instructions for performing the assays or diagnostic methods.

Other features and advantages of the present invention will be better understood by reference to the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. BRCA1 transfection inhibits DNA synthesis in human cancer cells. SW480 cells were cotransfected with pCR3, pCR3-BRCA1, pCEP4, or pCEP4-p53.

The percent BrdU(+)/GFP(+) cells shown was determined by analyzing at least 75 GFP(+) cells for each transfection in three fields, for each of two independent experiments.

Figure 2. BRCA1 transactivates the human and mouse p21-promoter and upregulates endogenous p21 protein expression. **Fig. 2A.** SW480 or HCT116 cells were cotransfected with pWWP-Luc and either pCR3 or pCR3-BRCA1 and luciferase activity was measured 24 hrs later as described in Example 1. **Fig. 2B.** CV1 cells were cotransfected pCAT1 and pCR3 or pCR3-BRCA1 and CAT activity was measured after 48 hrs later as described in Example 1. **Fig. 2C.** Structure of the human p21-promoter luciferase reporter and several 5'-deletions are shown schematically (left). The 5' end of each deletion is as indicated while the 3' boundary is 16 bp downstream of the p21 transcription initiation site (El-Deiry et al., 1993, Cell 75:817-825), fused to the luciferase reporter gene (El-Deiry et al., 1995, Cancer Res. 55:2910-2919). S1 and S2 indicate the relative locations of the two p53 DNA-binding sites within the 2.3 kb regulatory region upstream of the *WAF1/CIP1* gene (El-Deiry et al., 1995, *supra*). pWWP-Luc or the 5'-deletion mutants were cotransfected with pCR3 or pCR3-BRCA1 into SW480 cells and luciferase activity was assayed as in *Fig. 2A*.

Figure 3. BRCA1 mutants are defective for activation of p21. **Fig. 3A.** Structure of BRCA1 and various mutants used is shown schematically (left). Synthetic mutants lacking the functional nuclear localization signal (ΔNLS) (El-Deiry et al., 1995, *supra*), a ΔNLS mutant with a C-terminal fused NLS (ΔNLS/C+NLS), mutants lacking the RAD51-interacting domain (Δ515-1091), the C-terminal transactivation domain (Δ1314-1863) or both (Δ500-1863) are shown. The

corresponding extent of p21 induction (data from Figs. 3B and 3C) is shown (right; "++" = greater than 5-fold induction; a single arrow = 1.5- to 2-fold reduction in p21 activation; double arrow = substantial decrease in p21 activation, corresponding to less than 1.5-fold induction). **Fig. 3B**, COS-7; or **Fig. 3C**, SW480 cells were co-transfected with pWWP-Luc and pCR3 or either WT or mutant BRCA1 expression plasmids as indicated and luciferase activity was determined as in Fig. 2.

Figure 4. BRCA1 fails to inhibit DNA synthesis in $p21^{-/-}$ human cancer cells. The percent BrdU(+)/GFP(+) cells was determined following transfection of $p21^{+/+}$ (lanes 1,2) or $p21^{-/-}$ (lanes 3,4) HCT116 cells with pCR3 (lanes 1,3) or BRCA1 (lanes 2,4) as described in Fig.1.

Figure 5. BRCA1 stimulates *p53*-dependent transcription. **Fig. 5A,** SW480; **Fig. 5B,** MEF $p53^{-/-}$; or **Fig. 5C,** HCT116 cells were cotransfected with different combinations (as indicated by a A+") of *BRCA1* or its vector, pCR3 (3.5 μg) and *p53* or its vector, pCEP4 (2 μg) and PG13-LUC reporter (1 μg). **Fig. 5D**. MCF7 cells were transfected by either *BRCA1* or its vector, pCR3 (16 μg) and PG13-LUC reporter (4 μg). **Fig. 5E**. SW480 cells were transfected with the PG13-LUC reporter (0.5 μg) and increasing concentrations of *BRCA1* or its vector, pCR3 (1, 2, and 3.5 μg, respectively) either in the presence or absence of *p53* (0.1 μg). **Fig. 5F** and **Fig. 5G**. SW480 cells were cotransfected with different combinations (as indicated "+ ") of *BRCA1* or its vector, pCR3 (3.5 μg) and *p53* or its vector, pCEP4 (2 μg) along with 1 μg of either the *p21*-promoter WWP-LUC reporter (Fig. 5F) or the *bax*-promoter p*bax*-LUC reporter (Fig. 5G). Cells were harvested after 24 hrs of transfection and luciferase activity was measured as described in Example 2.

Figure 6. Schematic diagram identifying the p53-binding region of human BRCA1. The structure of BRCA1 protein is shown, including the nuclear localization signal (NLS), the transactivation domain (TA), the RING-finger domain (RING), the Rad51-interacting domain (Rad51) and the p53 binding domain (p53 binding). Mutants of BRCA1 tested for interaction with p53 are shown along with their ability to interact with p53 protein *in vitro* ("+" indicates interaction while "-" indicates no interaction).

Figure 7. Schematic diagram of human p53 identifying the BRCA1-interacting domain. The structure of the p53 protein is shown including the transactivation domain (TA), the DNA-binding domain, the tetramerization domain (TD) and the BRCA1-binding domain (bracket). Truncation mutants of p53 are shown along with their ability to interact with BRCA1 *in vitro* (right; "+" indicates interaction while "-" indicates no interaction).

Figure 8. Transactivation-deficient BRCA1 mutants are defective in stimulation of *p53*-dependent transcription. **Fig. 8A.** SW480 cells were cotransfected with the PG13-LUC reporter (1 μg), *p53* (2 μg) and either wt BRCA1 or its mutant derivatives (3.5 μg) as indicated "+". **Fig. 8B.** SW480 cells were transfected with the -143-LUC *p21* reporter (1 μg) and different combinations (as indicated "+") of *BRCA1* or its vector, pCR3 (2 μg) and *p53* or its vector, pCEP4 (2 μg) along with 1 μg of -143-LUC *p21* reporter. **Fig. 8C** and **Fig. 8D.** Inhibitory negative effect of *BRCA1* deletion mutant on *p53* transcription activity. **Fig. 8C.** SW480 cells were transfected with 0.5 μg of the PG13-LUC reporter and different combinations (as indicated "+") of pCEP4 or *p53*

(0.25 μg) and increasing amounts (lane 3, 2 μg; lane 4, 4 μg; and lane 5, 8 μg) of *BRCA1* Δ500-1863. The total amount of DNA was maintained constant at 8.75 μg by adding pCR3 DNA. **Fig. 8D**, SW480 cells were transfected with 0.5 μg of the PG13-LUC reporter and different combinations (as indicated "+") of pCEP4 or *p53* (0.25 μg), *BRCA1* Δ500-1863 (2 μg) and increasing amounts (lane 4, 4 μg; lane 5, 6 μg) of wt *BRCA1*. The total amount of DNA was kept constant at 8.75 μg by adding pCR3 DNA. Cells were harvested 24 hrs later and luciferase activity was measured as described in Example 2.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specifications and claims.

With reference to nucleic acid molecules, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA

molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to proteins or peptides, the term "isolated protein (or peptide)" or "isolated and purified protein (or peptide)" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. For purposes of this invention, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program are the parameters intended to be used herein to compare sequence identity and similarity.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the protein (i.e. the structure, thermostability characteristics and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of

the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

With respect to antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

With respect to oligonucleotides or other single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The term "promoter region" refers to the transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

The term "operably linked" means that the regulatory sequences necessary for expression of a particular coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other regulatory elements (e.g., enhancers or translation regulatory sequences) in an expression vector.

II. Description

A significant proportion of hereditary breast and ovarian cancer predisposition has been attributed to inherited defects in the *BRCA1* tumor suppressor gene. BRCA1 is a nuclear protein with properties of a transcription factor, and can interact with the recombination and repair protein RAD51. Young women with germline alterations in BRCA1 develop breast cancer at rates 100-fold higher than the general population and BRCA1-null mice die before day 8 of development, but the mechanisms of BRCA1-mediated growth regulation and tumor suppression heretofore has remained unknown.

In accordance with the present invention, it has now been shown that BRCA1 transactivates expression of the CDK-inhibitor $p21^{WAF1/CIP1}$ in a p53-independent manner and that BRCA1 inhibits S-phase cell cycle progression following its transfection into human cancer cells. BRCA1 does not inhibit S-phase progression in p21-/- as compared to p21+/+ cells and tumor-associated transactivation-deficient mutants of BRCA1 are defective in both transactivation of p21 and cell cycle inhibition. Thus, one mechanism by which BRCA1 contributes to cell cycle arrest and growth suppression is through p53-

independent induction of p21.

Deletion mapping of the human *p21* promoter identified a control region of 50 base pairs within the proximal promoter, -143 to -93 of the human *p21*, that mediates activation of p21 by BRCA1 (Fig. 2C). Deletion mapping further revealed that the two previously described p53 binding sites were not required for BRCA1 transactivation of p21.

The inventors have also discovered that BRCA1 increases p53-dependent transcription from the *p21* and *bax* promoters and that BRCA1 and p53 proteins interact both *in vitro* and *in vivo*. The interacting regions map, *in vitro*, to amino acid residues 224-500 of human BRCA1 (SEQ ID NO:1) and the C-terminal domain of p53 (residues 300-393 of SEQ ID NO:2). Tumor-derived transactivation deficient BRCA1 mutants are defective in co-activation of p53-dependent transcription and a truncation mutant of BRCA1 that retains the p53-interacting region acts as a dominant inhibitor of p53-dependent transcription. BRCA1 and p53 cooperatively induce apoptosis of cancer cells. These results indicate that BRCA1 and p53 coordinately regulate gene expression in their role as tumor suppressors.

The discoveries made in accordance with the present invention can be utilized for a variety of purposes, including but not limited to (1) assay systems to identify pharmacological agents capable of suppressing or enhancing cell proliferation by affecting the p53-dependent or -independent BRCA1-mediated transcription pathways described above; (2) diagnostic methods for assessing functional mutations in BRCA1 and /or p53 relating to their ability to associate with each other or with *p21* regulatory regions or regulatory regions of

other downstream targets involved in cell cycle arrest or apoptosis; and (3) methods for controlling cell proliferation by regulating either cell cycle progression or apoptosis via p53-dependent or -independent BRCA1-mediated transcription pathways, particularly *via* p21.

The following description sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1999) (hereinafter "Ausubel et al.") are used.

III. Biological Molecules and Cells

In addition to cells and biological molecules used for general cloning and expression, the following biological molecules and cell types are either required or useful for practice of the methods of the present invention: (1) cloning or expression vectors comprising nucleic acids encoding BRCA1 and p53, or specific portions thereof; (2) reporter genes operably linked to promoters responsive to p53 and/or BRCA1; (3) isolated BRCA1 or p53 protein, or selected portions thereof; (4) antibodies immunologically specific for BRCA1, p53 or selected portions thereof; and (5) various cell lines, including lines deficient in functional p53, BRCA1 (or both), or deficient in downstream targets of p53 or BRCA1, and further engineered with specified reporter genes.

Any nucleic acid molecule that encodes BRCA1 or p53 is contemplated for use in the present invention. Likewise, any BRCA1 or p53 protein is considered suitable for use in the present invention. Human BRCA1 and p53 are exemplified herein (e.g., SEQ ID NO:1 and SEQ ID NO:2). Another preferred source of BRCA1 and p53 proteins and nucleic acids is the mouse. Any other BRCA1 and p53 nucleic acids also may be used. These genes and proteins have been extensively characterized, and sequence information is widely available, e.g., through GenBank and the published and patent literature. For instance, U.S. Patent No. 5,654,155 discloses a consensus DNA sequence for the *BRCA1* gene. U.S. Patent 5,750,400 discloses three *BRCA1* coding sequences; the following Genbank Accession Numbers contain BRCA1 protein sequences: 1147603, 1418288, 3994048, 3994047, 3994046, 3209867, 2489823, 1698399, 728984, 627392, 1620568.

Included in the present invention is a nucleic acid molecule comprising the region of the proximal promoter of the *p21* gene responsible for p53-independent BRCA1 transactivation of *p21*. Although the BRCA1-responsive element from *Homo sapiens* is described and exemplified herein (-143 to -93 from the transcription start site noted in SEQ ID NO:3), this invention is intended to encompass the BRCA1 response element of the *p21* promoter from other mammals that are sufficiently similar to be used instead of the *Homo sapiens* nucleic acid sequence for BRCA1-mediated transactivation. These include, but are not limited to, allelic variants and natural mutants in the relevant region of SEQ ID NO:3 (bases 4441-4491), as well as homologs found in different species of mammals. Because such variants are expected to possess certain differences in nucleotide sequence,

this invention provides an isolated nucleic acid molecule having at least 60% (preferably 70% and more preferably over 80%) sequence homology to the nucleotide sequence set forth as residue -143 extending to the transcription start site noted in SEQ ID NO:3 over the length of those residues (residues -143 to -93 comprise the specific element conferring transactivation by BRCA1; however, the intervening 5' promoter region comprises regulatory elements needed for transcription (for instance, the TATA box). Because of the natural sequence variation likely to exist among BRCA1 transactivated genes, one skilled in the art would expect to find up to about 30-40% nucleotide sequence variation, while still maintaining the unique properties of the BRCA1 response element of the human *p21* promoter.

Peptides comprising the regions of BRCA1 and p53 constituting the protein-protein binding site, and nucleic acids encoding those peptides, are included in the present invention. As binding sites do not require absolute conservation of sequence and a some substitution of amino acids of similar chemical characteristics is allowed, the invention is contemplated to include peptides comprising sequences similar residues 224 to 500 of SEQ ID NO:1 or residues 300-393 of SEQ ID NO:2. In a preferred embodiment, peptides are 80% similar to residues 224-500 of SEQ ID NO:1 or residues 300 to 393 of SEQ ID NO:2 over the length of aa residues 224 to 500 of SEQ ID NO:1 or residues 300-393 of SEQ ID NO:2; (more preferably peptides are 90% similar, and most preferably peptides are 95% similar).

The use of the p53-binding sites to confer or remove p53 activation and/or p53/BCRA1 coactivation upon promoters is considered to be part of the invention. The

removal of the p53 binding sites from p53-regulated promoters both *in vitro* and *in vivo* will allow these promoters to be used in constructs without p53 regulation as well as to create cell lines with altered p53 regulation. Promoters without p53 regulation may be changed to p53 regulation through the inclusion of p53-binding sites. It is further contemplated that variations of the p53-binding site sequence may be used for this invention.

The use of BCRA1 sequence without the transactivation domain but with the p53 binding domain on BRCA1 to lessen p53 regulation cell proliferation is considered part of the invention. In a preferred embodiment, the region of BCRA1 with the p53-binding domain and without the transactivation domain is used. In accordance with this aspect of the invention it is now known that the BRCA1/p53 interaction will be hindered by the addition of partially functional binding domains, so it is highly likely that the BRCA1 binding domain on p53 will likewise repress the BRCA1/p53 coactivation of genes. In another preferred embodiment, the region of p53 with the BRCA1 binding domain (residues 300 to 393 of SEQ ID NO:2) without the DNA-binding domain is used. In a more preferred embodiment, the p53 binding domain on BRCA1 (residues 224 to 500 of SEQ ID NO:1) or the BRCA1 binding domain on p53 (residues 300-393 of SEQ ID NO:2) is used. In a most preferred embodiment, the truncated BCRA1, BRCA1 Δ500-1863 (residues 1 to 499 of SEQ ID NO:1), is used. It is contemplated that proteins significantly similar to these proteins can also be used in this invention. In a preferred embodiment, proteins 60% similar to residues 1-499 of SEQ ID NO:1 are used, in a more preferred embodiment proteins 70% similar to

residues 1-499 of SEQ ID NO:1 are used, and in a most preferred embodiment, proteins 80% similar to residues 1-499 of SEQ ID NO:1 are used.

Nucleic acid molecules used in the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art. In addition, some of the nucleic acid molecules may be commercially available.

Proteins used in the present invention may be prepared in a variety of ways, according to known methods. The protein may be purified from appropriate sources, e.g., human or animal cultured cells or tissues, by immunoaffinity purification. However, this is not a preferred method due to the low amount of protein likely to be present in a given cell type at any time.

The availability of nucleic acids molecules encoding BRCA1, p53 and portions thereof enables production of the protein using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription vector, such a pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

Alternatively, according to a preferred embodiment, larger quantities of protein may be produced by expression in a suitable procaryotic or eucaryotic system. For example, part or all of a DNA molecule may be inserted into a plasmid vector adapted for expression in a bacterial cell, such as *E. coli*, or into a baculovirus vector for expression in an insect cell.

Such vectors provide the regulatory elements necessary for expression of the DNA in the given host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

Proteins produced by gene expression in a recombinant procaryotic or eucyarotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

Some aspects of the present invention also utilize antibodies which bind immunospecifically to BRCA1 or P53 epitopes or proteins. Such antibodies may be prepared according to standard methods, if they are not commercially available. In a preferred embodiment, monoclonal antibodies are prepared, which react immunospecifically with various epitopes of BRCA1 or p53. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols. Polyclonal or monoclonal antibodies that immunospecifically interact with BRCA1 or p53 can be utilized for purifying the proteins, according to standard methods. They also may be used in the diagnostic and therapeutic methods described below.

Vectors that express the BRCA1 and p53

proteins, protein fragments, mutants, etc. are used in a variety of assays of the invention, as described below. For instance, the examples teach the transfection of cells with pCEP4-p53 and pCR3-BRCA1 expression vectors. Any vector that expresses the desired coding sequence in adequate amounts in the cell line of choice is acceptable. If non-*Homo sapiens* cell lines are used, different expression vectors may be required to achieve adequate expression of the protein, as is well known in the art. Many different kinds of expression vectors are commercially available (e.g., from Invitrogen).

Reporter genes operably linked to BRCA1- or p53-responsive promoters are also used in certain of the assays described below. Examples of several such reporter plasmids specific to p53/BRCA1 activation are taught in Examples 1 and 2. PG13-LUC, p*bax*-LUC and pWWP-LUC are all reporter plasmids that express luciferase. The use of the luciferase as a reporter molecule is well known in the art. The aforementioned reporter plasmids use three different promoters that are all coactivated by BRCA1/p53. The p21 promoter of the pWWP-LUC reporter plasmid alone exhibits p53-dependent and p53-independent coactivation. The use of pWWP-LUC plasmid is therefore particularly suited to address how a test molecule may differentially affect these two aspects of BRCA1 activation. Other reporter genes may be expressed, and many are commercially available. Reporter genes of interest include, but are not limited to, those encoding green fluorescent proteins (GFPs), β-glucuronidase (GUS) and β-galactosidase, among others.

A variety of cell types can be used to practice different aspects of the present invention. In the assays described below for screening potential

pharmacological agents, cell lines deficient in one or more of BRCA1, p53 or transactivational targets of BRCA1 and/or p53, are preferred. For instance, Example 2 teaches that SW480 human colon adenocarcinoma cells and mouse embryo fibroblast cells may be used. In general, tumor cells will provide the best source of regulation-deficient lines for use in the present invention. However, as discussed below, normal cells may also be used. For instance, to expand a normal cell line, the BRCA1-mediated transactivation of tumor suppressor genes would be down-regulated in order to stimulate proliferation of the cells.

Cells lines additionally do not need to be from humans or mice, but may be from other mammalian sources, such as rats, pigs, dogs, cows and primates, to name a few. Other useful cell lines and systems for these assays include, but are not limited to, cell lines derived from *Drosophila melanogaster* or *Caenorhabditis elegans*.

IV. Methods and Kits

Provided in accordance with the present invention are assay systems that can be used to discover and develop pharmacological agents capable of affecting the regulation of BRCA1-activated gene transcription, and their use in the treatment and control of cell proliferative disorders. The assay systems can be used in their entirety in cells, or partially *in vitro*.

One class of assays of the invention are assays for molecules that participate in the BRCA1/p53 cell proliferation regulation pathway. These assays use the BRCA1/p53 interaction as a basis for determining the degree of specific physical interaction that a test

molecule has with p53 and/or BRCA1, and the effect of the test molecule will have on the cell cycle progression. These assays may be performed *in vitro* and *in vivo*.

One assay of this type is an *in vitro* assay that determines the effect of test molecules on the protein-protein interaction between p53 and BRCA1. While this assay has many embodiments, the basic assay consists of three steps:

1. Incubating BRCA1 and p53 in the presence or absence of the test molecule;

2. Separating complexes comprising p53 and BRCA1 from the unbound molecules; and

3. Determining the effect of the test molecule on the formation of complexes comprising p53 and BRCA1.

Each of these step has many variations, the choice of which will customize the assay for a particular use.

In step 1, BRCA1 and p53 may be provided as isolated proteins (e.g., produced separately by *in vitro* transcription/translation or by *in vivo* expression). Alternatively, vectors that express genes encoding the proteins may be utilized. The test compound can be any of a variety of substances, including but not limited to small molecules, peptides, proteins, nucleic acids, or any combination thereof.

In step 2, numerous variations exist, the choice of which depends on the laboratory facilities and skills available. Example 2 teaches the use of glutathione-sepharose beads to precipitate glutathione S-transferase (GST) fusion proteins. Example 2 also teaches the use of antibodies specific to p53 or BRCA1 to precipitate complexes. Many variations exist on these two methods. Kits are commercially available with

different kinds of beads and methods to bind to proteins of interest. These beads may be separated from the total assay buffer by centrifugation, filtering, or in the case of magnetic beads, magnetism. Numerous methods exist that will be well known and usable by those skilled in the art.

Step three determines the molecules that are in the isolated complexes. If a non-protein test molecule has been employed in step one, the outcome in step three may be that BCRA1 does not associated with p53 as well as the controls, or BCRA1 may associate with p53 better than the controls. In Example 2, SDS-PAGE is taught as a method for identifying the components of the complexes by molecular weight. If the protein molecules of the assay are labeled with [^{35}S]-methionine as is taught in Example 1, then they can be identified by fluorography. [35S]-methionine labeling additionally allows the amount of the protein to be accurately determined and allows the detection of levels of protein too low for Coomassie staining and silver staining after SDS-PAGE. Additionally, proteins in the precipitated complex can be identified by western blotting after PAGE according to methods well known to those skilled in the art. Anti-BRCA1 and anti-p53 monoclonal antibodies are commercially available (Oncogene Science).

Another assay of the invention is a cell-based assay to determine the effect of a particular test molecule on p53-dependent and p53-independent BRCA1 regulation of cell proliferation regulation pathways. In accordance with the present invention it has been learned that BRCA1 regulates the cell cycle through p53-dependent coactivation and p53-independent activation of genes. While this assay has many embodiments, the basic assay

consists of two steps:

1. Cotransfection of a cell line that lacks functional p53 and BRCA1 with a series of vector combinations that express p53, BRCA1, and empty vector controls, for instance, such as those shown in the following array, along with a reporter gene operably linked to a promoter activated by p53 and/or BRCA1:

BCRA1 expression vector	-	+	-	+	-	+	-	+
empty BRCA1 expression vector	+	-	+	-	+	-	+	-
p53 expression vector	-	-	+	+	-	-	+	+
empty p53 expression vector	+	+	-	-	+	+	-	-
Test molecule	-	-	-	-	+	+	+	+

2. Determine the level of activity of the BRCA1/p53 cell proliferation pathway.

Each of these steps has numerous variations, the choice of which will customize the assay to specific conditions. This assay can be used to particular advantage in conjunction with the *in vitro* assay. Test molecules that are found to inhibit the p53/BRCA1 interaction in the *in vitro* assay can be tested for their *in vivo* action in this cell-based assay.

In the first step, a cell line that is deficient in p53 and/or BRCA1 is used, and may be further engineered with reporter genes operably linked to p53-specific or BRCA1-specific promoters, as described for instance in Example 2. The transfection methods taught in Example 2 are lipofectin, lipofectamine, and calcium

phosphate-precipitation. The choice of transfection method depends on the type of cell used in the transfection, and such methods are well known to persons skilled in the art. Additionally, cell lines stably transformed with the appropriate expression cassettes may be used instead of transfection.

Step two of the cell based assay requires that the activity of the BRCA1/p53 cell proliferation pathway be determined. Two general methods are taught in the examples. Reporter genes may be co-transfected into cells. Alternatively, an apoptosis assay or cell cycle arrest assay may be used as a means of determining the activity of the BRCA1 or BRCA1/p53 cell proliferation pathways. The TUNEL assay is taught in Example 2 as a method for determining the degree of cell apoptosis. This method, and other methods that measure the gross outcome of BRCA1/p53 cell proliferation pathway regulation, are very useful for determining the therapeutic and industrial usefulness of test compounds. Other methods that measure this broad scope of effect include, but are not limited to, the BrdU incorporation assay to measure DNA synthesis taught in Example 1 and assays for activity of apoptosis-related enzymes, e.g., caspase. Various assays to measure cell proliferation, cell cycle arrest and apoptosis are known to persons skilled in the art.

The present invention also includes diagnostic assays. For instance, standard genetic screening for mutations in the p53/BRCA1 respective binding domains, or the domain responsible for p53-independent binding of BRCA1 to the *p21* promoter, is now possible in accordance with the present invention. Such screening assays may utilize the specific nucleic acid molecules as

hybridization probes. Alternatively, antibodies immunologically specific for the respective binding domains may be developed and utilized.

Nucleic acid molecules encoding BRCA1 and p53 (and specific portions thereof, as well as part or all of the proteins themselves, may be used to advantage to control cellular proliferation, either through p53-independent regulation of *p21* or through co-regulation of apoptosis signal transduction by BRCA1 and p53. In a preferred embodiment, the p53-independent BRCA1 activation pathway or the BRCA1/p53 co-activation pathway is up-regulated, thereby inducing cell cycle arrest or apoptosis. Such up-regulation is suitable for treatment of neoplastic cell growth either in cultured cells or *in vivo*, in a patient requiring such therapy.

In another embodiment, the aforementioned pathways are down-regulated, resulting in an increase in cell proliferation due to the removal of cell cycle checkpoints and apoptosis signal transduction. This type of down regulation is useful for expanding cell lines in culture, or as a research tool to study the effect of such manipulation on long term cell growth and development.

Up-regulation or down-regulation of p53-independent or -dependent BRCA1-mediated transcription may be accomplished in a variety of ways known to persons skilled in the art. Some of these ways are described above, and utilize various DNA or protein fragments, or truncation mutants of BRCA1, to block the pathway(s) at various points, thereby down-regulating the pathway(s) and increasing cell proliferation. Other methods include, but are not limited to, (1) over-producing BRCA1 or p53 in cells that do not produce the proteins, or that

produce defective proteins; and (2) enhancing the interaction between BRCA1 and p53 or the *p21* promoter. Such methods are considered within the scope of the present invention.

According to another aspect of the invention, kits are provided to facilitate performing the above-described assays. In one embodiment, the kits comprise one or more of the DNA constructs encoding BRCA1, p53, fragments and mutants thereof, and reporter genes operably linked to transactivational targets of BRCA1 and/or p53, as described in greater detail herein, along with instructions on how to use the constructs to perform the assays of the invention In another embodiment, the kits comprise aliquots of transgenic cells and instructions for their use. In another embodiment, the kits may comprise antibodies and other reagents for performing immunological assays. The kits may also comprise, optionally, various additional reagents for the assays, such as growth media, enzyme substrates for the reporter gene product, and standard solutions for calibrating expression of the reporter gene.

The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

EXAMPLE 1
BRCA1 Arrests the Cell Cycle by Transactivating the Expression of $p21^{WAF1/CIP1}$

Because several known tumor suppressor genes interact with or negatively regulate the cell cycle machinery, the effect of BRCA1 on cell cycle progression was investigated. This example describes the results of that investigation.

A. Materials and Methods

Plasmid constructs. The pCEP4-p53 and pWWP-Luc plasmids are described in the literature (El-Deiry et al., 1993, *supra*). The construction of the p21-promoter deletions fused to the luciferase reporter gene has been described (Zeng et al., 1997, Nature Genetics 15:78-82). The human p21-promoter luciferase-reporters with 5'-truncations at -153, -143 and-133 were constructed and sequenced as described (Zeng et al., 1997, Nature Genetics 15:78-82). The murine p21 promoter-CAT reporter, pCAT1, has been described (El-Deiry et al., 1995, Cancer Res. 55:2910-2919). The GFP expression vector pGreen Lantern-1 was obtained from Gibco. The pCR3-BRCA1 expression plasmid has been previously described (Thankur et al., 1997, Mol. Cell. Biol. 17:4444-452).

pCR3 vectors encoding synthetic and tumor-associated BRCA1 mutants (Fig. 3A) were constructed as follows. The RAD51-interaction-deficient mutant plasmid (Δ515-1091) was constructed by digestion of pCR3-BRCA1 using *Bsu36*I to delete nucleotides 1661-3392 (Miki et al., 1994, Science 266:66-71), followed by in-frame intramolecular ligation. The transactivation domain deletion mutant plasmid (Δ1314-1863) was prepared by digestion of pCR3-BRCA1 with *Bam*H1 (nucleotide 4058) and *Not*1 (nucleotide 5833), Klenow fill-in and subsequent intramolecular blunt-end ligation at 37°C. The double mutant (Δ500-1863) was cloned by polymerase chain reaction (PCR) amplification of nucleotides 1-1616 using pCR3-BRCA1 as a template and the following primers:

5'-GCAAGCTTGCCACCATGGATTTATCTGCTCTTCGC-3' (SEQ ID NO:4) and

5'-TTGTGAGGGGACGCTCTTGTA-3' (SEQ ID NO:5).

The 1.6-kilobase PCR product was ligated into the pCR3 vector, and a clone expressing the N-terminal region of BRCA1 in the sense orientation downstream of the CMV-promoter was isolated. The ΔNLS/C + NLS vector was prepared as follows. A 488-bp DNA fragment containing a fusion between the extreme C terminus of BRCA1 and the NLS region (amino acids 499-510) was generated by PCR amplification of pCR3-BRCA1 using the following primers:

5'-AGGAGATGTGGTCAATGGAAG-3' (SEQ ID NO:6) and

5'-TATCATGATGTAGGTCTCCTTTTACGCTTTAATTTATT GTAGTGGCTGTG-3' (SEQ ID NO:7).

This PCR product was subcloned into pCR3 and released as an *Apa*1 fragment which was cloned in-frame into an *Apa*1-digested ΔNLS plasmid. pCR3 vectors encoding the transactivation-domain tumor-derived BRCA1 mutants (Chapman and Verma, 1996, *supra*; Monteiro et al., 1996, *supra*) (P1749R, Y1853insA, Q1756insC) were constructed by amplification of a 1.8-kb C-terminal region containing the specific mutations and subcloning into pCR3-BRCA1 digested with *Bam*H1 and *Not*1. All mutant BRCA1 expression plasmids used were sequenced and shown to express protein. Deletion of the nuclear localization signal (Thakur et al., 1997, *supra*) (ΔNLS mutant) resulted in cytoplasmic staining, which reverted back to the nucleus upon addition of a C-terminal NLS (ΔNLS/C + NLS; unpublished data).

Cells, transfections and luciferase assays. The $p21^{+/+}$ parental and $p21^{-/-}$ HCT116 human colon cancer cells (Waldman et al., 1995, Cancer Res. 55:5187-5190) were provided by T. Waldman and B. Vogelstein. SW480, HeLa, COS-7 and CV1 cells (ATCC) were transfected as described (El-Deiry et al., 1993, Cell 75:817-825). Luciferase and CAT assays were performed as described

(El-Deiry et al., 1993, *supra*; El-Deiry et al., 1995, Cancer Res. 55:2910-2919). HeLa cells expressing BRCA1 or mutants were obtained following transfection of HeLa cells with BRCA1, ΔNLS or Δ515-1091 mutants and continuous growth in 0.4 $mg*ml^{-1}$ G418.

Analysis of BrdU incorporation in transfected GFP(+) cells. SW480 or HCT116 cells were co-transfected with pGreen Lantern-1 and mammalian expression vectors (as indicated in Figs 1,4 and Table 1) at a ratio of 1:3. At 12 h after transfection, BrdU (Sigma) was added at a final concentration of 20 μM and the cells incubated for 20 h at 37°C. Cells were examined by fluorescence microscopy to identify GFP(+) cells. Cells were treated with a mouse anti-BrdU monoclonal antibody (BM 9318, Boehringer Mannheim) and Rhodamine-conjugated goat anti-mouse IgG (Pierce) as described(Zeng et al., 1997, *supra*). The number of BrdU(+) cells was determined for all of the GFP(+) cells in three high-power fields, as described in Fig. 1.

Immunocytochemistry and immunofluorescence. SW480 cells, transfected with expression plasmids, were stained 24 h later with an anti-human-WAF1 monoclonal antibody (Ab1; Calbiochem) as described (El-Deiry et al., 1995, *supra*). Immunofluorescence analysis of BRCA1 expression was performed as described (Thakur et al., 1997, *supra*).

Northern blot analysis. Total RNA was isolated and northern blot analysis was performed as described (El-Deiry et a., 1993, *supra*), and p21 mRNA expression was detected using a 2.1-kb human p21 cDNA probe (El-Deiry et al., 1993, *supra*). Equivalent loading of various RNA samples was demonstrated using a probe for rpl32, which encodes a ribosomal protein(Meyuhas and

Perry, 1980, Gene 10:113-129).

Immunoprecipitation and western blot analysis. Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM EDTA, pH 8.0, 1 mM phenylmethylsulphonyl fluoride (Gibco), 1% antipain (Sigma), 1% leupeptin (Sigma), 1% pepstatin A (Sigma), 1% chymostatin (Sigma) and 1% AEBSF (Calbiochem). Immunoprecipitations were carried out in the lysis buffer using 2 μg of anti-BRCA1 monoclonal antibody (Ab1; Calbiochem) for 2 h at 4°C, followed by the addition of 50% protein A-agarose beads (Sigma) and incubation for 1 h. After 3 washes with lysis buffer the immunoprecipitated proteins were analyzed by western blotting as described in Example 1, using a 1:250 dilution of the anti-BRCA1 monoclonal antibody, which was raised using the N-terminal portion of recombinant human BRCA1 (amino acids 1-304) as the immunogen (Calbiochem).

B. Results

By using green fluorescent protein (GFP) to mark specific transfected cells (Zeng et al., 1997, Biotechniques 23:88-94), we examined the effects of *BRCA1* transfection on new DNA synthesis in SW480 and HCT116 human colon cancer cells (Figs 1, 4 and Table 1). We found fewer BrdU(+)/GFP(+) SW480 cells following transfection of either BRCA1 or p53 than with their control vectors (Fig. 1). A quantitative summary of the percentage of BrdU(+)/GFP(+) cells from three independent experiments in SW480 cells is shown in Fig. 1 and Table 1. BRCA1 inhibited new DNA synthesis in SW480 cells by approximately 50% compared with the pCR3 vector. The extent of inhibition of BrdU incorporation following BRCA1 transfection was similar to p53 transfection (Fig. 1). BRCA1 also inhibited S-phase progression in HCT116

cells (Fig. 4). These results suggest that BRCA1 can inhibit S-phase progression and thus negatively regulate the cell cycle in human cancer cells.

Table 1. Cell cycle effects of tumor-derived BCRA1 mutants.

	GFP(+) cells	BrdU(+) cells	BrdU(+)/GFP(+) (%)
pCR3	43	27	62.8
BRCA1	55	19	34.6
P1749R	59	31	52.5
Y1853insA	56	32	57.1

These BRCA1 mutants are defective at inhibiting DNA synthesis. GFP was used as a marker for transfection of SW480 cells. GFP(+) cells were examined for BRrdU incorporation (BrdU(+)) by anti-BrdU staining. BrdU(+)/GFP(+) cells with active DNA synthesis.

We investigated cyclin-dependent kinase (CDK) inhibition of cell-cycle progression as a potential mechanism by which BRCA1 may control cell proliferation. Induction of $p21^{WAF1/CIP1}$ expression has been linked to growth inhibition by p53 (El-Deiry et al., 1993, Cell 75:817-825)), and p21 expression also has been found to signal growth arrest, independent of p53, in cells undergoing differentiation (Zhang et al., 1995, Cancer Res. 55:668-674). The protein p21 is a universal cell-cycle inhibitor that specifically binds cyclin-CDK complexes and proliferating cell nuclear antigen, thereby serving as a potent growth inhibitor and effector of cell-cycle checkpoints (Sherr and Roberts, 1995, Genes Dev. 9:1149-1163). As BRCA1 contains a carboxy-terminal transactivation domain (Chapman and Verma, 1996, Nature 382:678-679), we hypothesized that BRCA1 may transcriptionally induce p21 expression and thus

negatively regulate cell-cycle progression. We examined the effect of BRCA1 on p21-promoter reporter gene expression following transfection into SW480, HCT116, COS-7, HeLa and CV1 cells (Figs 2, 3, and data not shown). BRCA1 activated the human p21 promoter luciferase-reporter by 5- to greater than 20- fold in SW480 (Fig. 2A), HCT116 (Fig. 2A), HeLa (data not shown) and COS-7 (Fig. 3B) cells, as compared to transfection of the pCR3 vector. BRCA1 also transactivated the mouse p21-promoter by more than 10-fold in CV1 cells (Fig. 2B).

Deletion mapping within the human p21 promoter identified a control region of 50 base pairs (between -143 and -93) within the proximal promoter that seems to mediate activation of p21 by BRCA1 (Fig. 2C). The two p53-binding sites are not required for BRCA1 transactivation of p21. Whether p21 activation by BRCA1 is a direct consequence of BRCA1 binding to the p21 promoter or is an indirect effect is not known. We also investigated whether BRCA1 could activate endogenous p21 mRNA and protein expression. Figure 3D shows that p21 mRNA levels were elevated in HeLa cells after BRCA1 transfection. By using immunochemical methods, we also found increased levels of endogenous p21 protein in SW 480 cells transfected with BRCA1, compared with cells transfected with vector alone. Thus, transcriptional activation of p21 by BRCA1 appears to be functionally relevant.

To further elucidate the biological importance of p21 regulation by BRCA1, we examined the effect of various synthetic and tumor-associated mutant BRCA1 proteins on p21 expression and cell-cycle progression (Figs. 3,4 and Table 1). Mutants of BRCA1 lacking a functional nuclear localization signal, the C-terminal

transactivation domain, the RAD51-interacting domain or all three domains were deficient in activating p21 expression (Fig. 3A,B). Similarly, three different tumor-associated transactivation-deficient (Chapman and Verma, 1996, *supra*; Monteiro et al., 1996, PNAS 93:13595-13599) BRCA1 mutants were defective in activating the human p21-promoter luciferase-reporter gene (Fig. 3C). The two tumor-associated transactivation-deficient BRCA1 mutants tested for cell-cycle inhibition were also found to be deficient in cell-cycle inhibition in SW480 cells (Table 1). These results indicate that transactivation by BRCA1 may be required for its cell-cycle inhibitory effect, and that tumor-derived BRCA1 mutants may be defective in cell-cycle inhibition.

To determine whether p21 is required for the cell-cycle inhibitory effect of BRCA1, we examined the extent of new DNA synthesis following BRCA1 transfection into $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells. BRCA1 inhibited new DNA synthesis in $p21^{+/+}$ HCT116 cells, but there was no evidence of DNA synthesis inhibition resulting from BRCA1 in the $p21^{-/-}$ cells (Fig. 4). These observations indicate that p21 induction may be required for cell-cycle inhibition by BRCA1 in HCT116 cells. Expression of p21 has previously been shown to be required for cell-cycle arrest following γ-irradiation of these cells (Waldman et al., 1995, Cancer Res. 55:5187-5190).

These results demonstrate that BRCA1 can negatively regulate the mammalian cell cycle, and suggest that this effect is at least partly mediated by the ability of BRCA1 to induce p21. Although previous studies have reported that expression of BRCA1 is cell-cycle dependent (Vaughn et al., 1996, Cell Growth Differ. 7:711-715; Gudas et al., 1996, 7:717-723; Chen et al.,

1996, Cancer Res. 56:3168-3172), our results demonstrate that BRCA1 can inhibit cell-cycle progression. The absence of this inhibition in $p21^{-/-}$ cells indicates that p21 expression maybe essential for BRCA1 to inhibit new DNA synthesis. Decreased cell-cycle inhibition by transactivation-deficient tumor-derived BRCA1 mutants is consistent with the idea that regulation of p21 by BRCA1 may contribute to growth control. In support of this, recent observations in the yeast *Saccharomyces cerevesiae* demonstrate that the C-terminal 303 amino acids of BRCA1 are sufficient to inhibit yeast colony formation, and that tumor-associated mutations in the context of the 303 amino-acid region fail to inhibit colony growth (Humphrey et al., 1997, PNAS 94:5820-5825).

The results reported in this example are at odds with recent observations that cells from BRCA1-null mouse embryos have increased levels of p21 mRNA, which suggest that BRCA1 may suppress p21 expression during development to allow cell growth (Haken et al., 1996, Cell 85:1009-1023). However, p21 protein level and its effect on the cell cycle have not been determined in BRCA1-null embryos, and the mechanism of increased p21 expression remains unclear (Haken et al., 1996, *supra*). BRCA1 may serve different functions during development and adulthood. It also is possible that the absence of BRCA1 in these cells perturbs a feedback loop controlling expression of p21. Although our data do not provide a clear explanation for this difference, our results demonstrate that BRCA1 can transcriptionally induce p21 expression and negatively regulate the cell cycle. The identification of BRCA1 as an RNA polymerase II holoenzyme-associated protein provides additional evidence for the role of BRCA1 in transcriptional

activation (Scully et al., 1997, PNAS 94:5605-5610). The importance of this role in tumor suppression is further supported by the fact that about 90% of the mutations in *BRCA1* result in C-terminal truncations that involve the transactivation domain. The loss of cell-cycle inhibition in p21-/- cancer cells and the deficiency in p21 activation and cell-cycle inhibition by tumor-derived BRCA1 mutants supports the notion that p21 expression may lead to a quiescent or growth-inhibited state, which may contribute to BRCA1-dependent tumor suppression.

EXAMPLE 2
BRCA1 Interacts with p53 to Regulate Transcription

In this example, the participation BRCA1 and p53 may participate in a common pathway of growth regulation is demonstrated. BRCA1 and p53 are shown to form stable complexes, both *in vitro* and *in vivo*. BRCA1 is shown to be a potent coactivator of *p53*-dependent transcription of the *p21* and *bax* genes.

A. Materials and Methods

Cell Lines and Culture Conditions. The SW480 human colon adenocarcinoma cell line was maintained in culture as described in Example 1. HCT116 human colon cancer cells (wt p53) were provided by Bert Vogelstein (Johns Hopkins University), and cultured as previously described (McDonald et al., 1996, Cancer Res. 56 :2250-2255). Mouse embryo fibroblasts ($p53^{-/-}$) were provided by Tyler Jacks (Massachusetts Institute of Technology). SAOS-2, Hela, COS7, HBL100, U20S and 293 cells were obtained from ATCC.

Reporters and Expression Plasmids. The pWWP-LUC, PG13-LUC and pCEP4-*p53* plasmids were provided

by B. Vogelstein (Johns Hopkins University). p*bax*-LUC was obtained from Karen Vousden (NCI, Frederick, MD). The -143 *p21* Luc reporter was described in Example 1. pGEX-5X1 was purchased from Pharmacia, and the pCR3 and pCR3.1 were purchased from Invitrogen. The Luciferase T7 control DNA was obtained from Promega. Wild-type and mutant BRCA1 (Δexon11, Δ500-1863, Δ1312-1863, P1749R, Y1853insA and Q1756insC) were described previously (Thakur et al., 1997, *supra*; Somaundaram et al., 1997, *supra*). The *p53* cDNA was amplified using the primers 5'-AAGCTTGCCACCATGGAGGAGCCGCAGTCA-3' (SEQ ID NO:8) and 5'-ATGCGGCCGCTCAGTCTGAGTCAG-3' (SEQ ID NO:9) and the human *p53* cDNA as template. The resulting PCR product was sub-cloned into pCR3.1. Using the human *BRCA1* cDNA as template, venous *BRCA1* fragments were amplified and cloned into pCR3.1 for *in vitro* translation. The primers used were:

For *BRCA1* exon 11 (224-1365):
5'-TAAGCTTGCCACCATGGCTGCTTGTGAATTT-3' (SEQ ID NO:10) and 5'-ACTCGAGTCATAAGTTTGAATCCAT-3' (SEQ ID NO:11);

BRCA1 (1-1056):
5'-GCAAGCTTGCCACCATGGATTTATCTGCTCTTCGC-3' (SEQ ID NO:12) and 5'-GGAGCCCACTTCATTAGTAC-3' (SEQ ID NO:13);

BRCA1(1-98):
5'-GCAAGCTTGCCACCATGGATTTATCTGCTCTTCGC-3' (SEQ ID NO:14) and 5'-ACCTGTGTCAAGCTGAAAAGCAC-3' (SEQ ID NO:15);

BRCA1 (91-500):
5'-AAGCTTGCCACCATGTGTGCTTTTCAGCTT-3' (SEQ ID NO:16) and 5'-TCTCGAGTCAATTTGTGAGGGGACG-3' (SEQ ID NO:17);

BRCA1(224-500):
5'-TAAGCTTGCCACCATGGCTGCTTGTGAATTT-3' (SEQ ID NO:18) and 5'-TCTCGAGTCAATTTGTGAGGGGACG-3' (SEQ ID NO:19).

For production of GST-p53 fusion proteins,

full-length and different fragments of *p53* were amplified using the following primers:

wt *p53*:

5'-GTCGAATTCGAGGAGCCGCAGTCAGAT-3' (SEQ ID NO:20) and

5'-ATGCGGCCGCTCAGTCTGAGTCAG-3' (SEQ ID NO:21);

p53 (1-43):

5'-GTCGAATTCGAGGAGCCGCAGTCAGAT-3' (SEQ ID NO:22) and

5'-ATGCGGCCGCCAAATCATCCATTGC-3' (SEQ ID NO:23);

p53 (1-200):

5'-GTCGAATTCGAGGAGCCGCAGTCAGAT-3' (SEQ ID NO:24) and

5'-TACTCGAGATTTCCTTCCACTCGGAT-3' (SEQ ID NO:25);

p53 (100-300):

5'-ATGAATTCCAGAAAACCTACCAG-3' (SEQ ID NO:26) and

5'-TACTCGAGGGCAGCTCGTGGTG-3' (SEQ ID NO:27);

p53 (201-393):

5'-ATGAATTCTTGCGTGTGGAGTATTTG-3' (SEQ ID NO:28) and

5'-ATGCGGCCGCTCAGTCTGAGTCAG-3' (SEQ ID NO:29);

p53 (300-393):

5'-ATGAATTCCCCCCAGGGAGCACT-3' (SEQ ID NO:30) and

5'-TGCGGCCGCTCAGTCTGAGTCAG-3' (SEQ ID NO:31).

The PCR products were cloned into the following restriction sites of the pGEX-5X1 GST vector: wt *p53* *Eco*RI/*Not*I; *p53* (1-43) *Eco*RI/*Not*I; *p53* (1-200) *Eco*RI/*Xho*I; *p53* (100-300) *Eco*RI/*Xho*I; *p53* (201-393) EcoRI/*Not*I; *p53* (300-393) *Eco*RI/*Not*I. To make the GST-BRCA1 (91-500) fusion protein, the *BRCA1* (91-500) product was generated by PCR from the human *BRCA1* cDNA with primers 5'-AGAATTCTGTGCTTTTCAGCTT-3' (SEQ ID NO:32) and 5'-TCTCGAGTCAATTTGTGAGGGGACG-3' (SEQ ID NO:33). After digestion with *Eco*RI and *Xho*I, the fragment was subcloned into pGEX-5X1. The cloned DNA sequence of all of the above vectors was verified.

Transfections and Luciferase Assays. SW480 and MEF cells were transfected using lipofectin and lipofectamine (GIBCO) respectively as described previously (El-Deiry et al., 1993, *supra*). SAOS2 cells were transfected using the calcium phosphate precipitation described in Example 1. Luciferase assays were performed as described (Zeng et al., 1997, *supra*).

GST-fusion Protein Purification. Various pGEX-5X1 fusion constructs were transformed into DH5α *E. coli* (GIBCO/BRL). The GST-p53 or GST-BRCA1 (91-500) proteins were produced following incubation of the bacteria with 0.1-1 mM IPTG at 30°C for 2-3 hours. The cell pellet was resuspended in PBS and sonicated. The soluble GST-fusion proteins were immobilized onto Glutathione Sepharose 4B beads (Pharmacia).

***In vitro* Interaction Between BRCA1 and p53.** *In vitro* translations were carried out in the presence of 40 μCi [^{35}S]-methionine (NEN) using TNT7 coupled reticulocyte lysate system (Promega). For *in vitro* binding assays, five to 20 μl of *in vitro* translated ^{35}S-labeled proteins or cell lysate from $1x10^7$ cells was incubated with glutathione-sepharose beads containing GST-fusion proteins from 10 ml bacterial cultures. After washing, the bound proteins were eluted with 30 μl SDS sample buffer and resolved by 7.5% SDS-PAGE followed by fluorography. In experiments where unlabeled cell extracts were used, after SDS/PAGE separation, the proteins were transferred to Hybond™P membrane (Amersham). Immunoblotting was carried out using anti-BRCA1 monoclonal antibody (SG11, 1:100 dilution; Oncogene Science) and ECL™ (Amersham).

TUNEL Assay. SW480 cells ($3x10^4$/ well) were transfected with mammalian expression vectors (1 μg) in

8-well chamber slides. The cells were fixed with neutral buffered formalin after 40 hrs and analyzed for fluorescence emission by TUNEL assay using the Apoptag Plus In Situ apoptosis detection kit-fluorescein (ONCOR) as previously described (Wu et al., 1997, Nat. Genetics 17:141-143). Briefly, fixed cells were incubated with digoxigenin-labeled nucleotides and TdT enzyme followed by washing with PBS and incubation with an anti-digoxigenin-FITC conjugate. In each experiment, the % of TdT(+) cells was determined by counting at least 300 cells from 5-10 random transfected fields.

Immunoprecipitations and Immunoblotting of BRCA1 and p53. Immunoprecipitation was carried out using an immunoprecipitation kit (Boehringer Mannheim). About $1x10^7$ cells were lysed in Wash Buffer 1 (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate plus protease inhibitor cocktail). After preclearing with protein G-agarose beads, lysates were mixed with antibodies (1 μg/ml) and protein G-agarose. The antibodies used are anti-BRCA1, (C20; Santa Cruz) and (SG11; Oncogene Science), anti-p16 (Ab1; Oncogene Science), anti-MDM-2 (Ab1; Oncogene Science) and anti-p53 (Ab-6; Oncogene Science). The beads were washed three times with Wash Buffer I, one time with Wash Buffer 2 (50 mM Tris, pH 7.5, 500 mM NaCl, 0.1% NP40, 0.05% sodium deoxycholate), and one time with Wash Buffer 3 (50 mM Tris, pH 7.5, 0.1% NP40, 0.05% sodium deoxycholate). Samples were solubilized in SDS-sample buffer by boiling for 5 minutes and separated by 7.5 % SDS-PAGE. After transferring, the membranes were incubated with anti-p53 antibody conjugated to HRP (1:200 dilution of p53DO1; Santa Cruz) or anti-BRCA1 antibody (1:200 dilution of SG11; Oncogene Science). A 1:300 dilution of anti-mouse

HRP (Amersham) was used to detect BRCA1.

B. Results

BRCA1 stimulates *p53*-dependent transcription. Because both p53 and BRCA1 can transactivate the *p21* promoter, we investigated the effects of BRCA1 on *p53*-dependent transcription. *BRCA1* either alone or in combination with *p53* was cotransfected into SW480 colon carcinoma cells together with a synthetic *p53*-specific reporter (PG13-LUC) (El-Deiry et al., 1993, *supra*). The expression of *BRCA1* in SW480 (endogenous mutant *p53*) had no effect on *p53*-specific reporter expression in the absence of exogenous wild-type p53 (Fig. 5A). As previously reported, transfection of *p53* in SW480 cells led to stimulation of the PG13-luciferase reporter (El-Deiry et al., 1993, *supra*). Unexpectedly, a dramatic increase in luciferase activity was observed following cotransfection of SW480 cells by *BRCA1* and wt p53 expression vectors (Fig. 5A). We confirmed the effect of *BRCA1* on *p53*-dependent transcription in MEF *p53*-/- (Fig. 5B), wild-type *p53*-expressing HCT116 (Fig. 5C), wild-type *p53*-expressing MCF7 breast cancer cells (Fig. 5D) and SAOS-2 (*p53*-null Osteosarcoma) cells (data not shown). The coactivation of *p53*-dependent transcription by *BRCA1* was dose-dependent with respect to *BRCA1* and required the presence of wild-type *p53* (Fig. 5E).

The transcriptional activity of p53 leads to increased expression of *p21* and *bax*, putative effectors of p53-mediated growth arrest and apoptosis respectively (Levine, 1997). *BRCA1* stimulated *p53*-dependent transcription by six- to eight-fold from the *p21* and *bax* promoters, respectively (Fig. 5F,G). As expected, expression of *BRCA1* alone activated the *p21* promoter (see Example 1). However, *BRCA1* did not activate the *bax*

promoter in the absence of exogenous wt *p53* (Fig. 5G, lane 2). Because of the increased expression of endogenous *p53* target genes in wt *p53*-expressing cells as compared to cells which lack wt *p53* (Wu et al., 1997, *supra*; El-Deiry et al., 1994, *supra*; Blasgosklonny et al., 1997, Intl. J. Oncology 11:1165-1170) and because of the lack of availability of *BRCA1*-null cell lines, it has been difficult to demonstrate that *BRCA1* co-activates expression of endogenous *p53* targets. It remains unclear whether endogenous *p53*- dependent *p21* expression requires *BRCA1*. In transfection experiments it is possible to limit the amount of *p53*, a potent transcription factor, to demonstrate the role of *BRCA1* as a coactivator. Because numerous studies have previously correlated the expression of endogenous genes with these reporters, we believe that the coactivation of *p53*-dependent transcription provides important insight into how *BRCA1* may regulate the *p53*-dependent growth inhibitory pathway.

BRCA1 interacts with p53. BRCA1 may regulate p53-mediated transcription either directly through protein-protein interaction or indirectly through association with other proteins whose function may stimulate p53-mediated transcription. To determine whether BRCA1 binds to p53 *in vitro*, we used either glutathione S-transferase (GST) or GST-p53 fusion protein bound to Glutathione sepharose beads to precipitate either *in vitro* translated or cellular BRCA1 proteins. Approximately 20% of input ^{35}S-labeled BRCA1 bound to GST-p53 protein but no BRCA1 bound to GST alone. ^{35}S-labeled Luciferase protein, a negative control, did not bind to either GST or GST-p53.

In a similar experiment, COS-7 cell extracts were mixed with beads containing GST or GST-p53 and the

bound proteins were analyzed by BRCA1 immunoblotting. BRCA1 specifically associated with GST-p53 but not GST. To investigate the interaction between BRCA1 and p53 proteins *in vivo*, immunoprecipitations followed by western blots were performed. In these experiments, an anti-p53 monoclonal antibody directly conjugated to horseradish peroxidase was used to detect p53 directly. The endogenous p53 of COS-7, Hela, U20S and SW480 cells co-immunoprecipitated with BRCA1. Although p53 protein is well known to be targeted for degradation by HPV-E6 protein in Hela cells, it was readily detectable in our co-immunoprecipitation assay. Interaction of p53 with CBP has also been demonstrated in Hela cells (Gu et al., 1997, Nature 387:819-822). We have consistently observed that endogenous BRCA1 could be specifically recovered in association with the exogenous GST-p53 fusion protein linked to agarose beads.

We have found no evidence for a BRCA1-dependent supershift of p53-DNA binding site electrophoretic mobility using two different anti-human BRCA1 antibodies. It is possible that the interaction between BRCA1 and p53 may not be stable enough for gel shift experiments, that BRCA1 is not required for the DNA binding by p53, or that BRCA1 may be present but concealed by other proteins. Although BRCA1 and p53 appear to associate both *in vitro* and *in vivo*, we cannot exclude the possibility that the interaction may be indirect.

A novel N-terminal domain of *BRCA1* exon 11 interacts with p53 *in vitro*. To identify the region of BRCA1 that interacts with p53, a series of BRCA1 deletion mutants (Fig. 6) translated *in vitro* in the presence of ^{35}S-methionine were individually mixed with GST-p53-bound affinity resin. The region of BRCA1 extending from aa

residues 224 to 500 was found to be sufficient for interaction with p53. This region corresponds to the N-terminal region of exon 11 of BRCA1. To further confirm the minimal BRCA10-interacting region, either GST or GST-BRCA1 fusion protein containing residues 74 to 500 of BRCA1, was mixed with *in vitro* synthesized full length p53 protein. p53 protein specifically bound to the GST-BRCA1, whereas luciferase protein, did not bind to either GST or GST-BRCA1. This conserved domain (Szabo et al., 1996, Hum. Mol. Genet. 5:1289-1298) has not been noted previously to play a role in BRCA1 function. It is notable that this region is missing from several alternatively spliced forms of BRCA1 (Thakur et al., 1997, *supra*).

BRCA1 binds to the C-terminal domain of p53. In order to identify the region of p53 (Ko and Prives, 1996, Genes Dev. 10:1054-1072) which binds to BRCA1, a series of p53 deletion mutants (Fig. 7) were generated as GST-fusion proteins and their ability to interact with *in vitro* translated full-length BRCA1 or exon 11 of BRCA1 protein was studied. The region of p53 between aa residues 300-393 was found to be sufficient for its interaction with BRCA1. These results suggest that the physical interaction between p53 and BRCA1 may underlie the BRCA1-dependent stimulation of p53-mediated transcription.

***BRCA1* mutants are deficient in stimulation of *p53*-dependent transcription.** To further explore the biological relevance of the stimulation of *p53*-dependent transcription by *BRCA1*, we studied the effect of various synthetic and tumor-derived mutant BRCA1 proteins on *p53*-dependent transcriptional activity. Mutants of *BRCA1* lacking the C-terminus transactivation domain or both the

C-terminus transactivation domain and the Rad51-interacting domain were poor augmenters of *p53*-mediated transcriptional activity (Fig. 8A). Similarly, four tumor-derived transactivation-deficient *BRCA1* mutants were defective in stimulation of *p53*-mediated transcription (Fig. 8A). These results suggest that an intact transactivation domain within *BRCA1* may be required for *BRCA1* to increase *p53*-dependent transcriptional activity.

BRCA1 regulates $p21^{WAF1/CIP1}$ through a p53-dependent as well as a p53-independent mechanism. *p53* had no effect on *BRCA1*-dependent activation of the *p21*-promoter in the absence of *p53* DNA-binding sites (Fig. 8B), suggesting that the effect of *BRCA1* on *p53* (Fig. 5) is specific to *p53*-up-regulated genes. Thus it is clear that *BRCA1* can activate *p21* by a *p53*-independent pathway that maps to a region distinct from the *p53* DNA binding site and can coactivate *p53*-dependent gene expression in general only in the presence of *p53* binding sites (Figs. 1, 4).

Dominant negative inhibition of p53-dependent transcriptional activity by a *BRCA1* truncation mutant retaining the p53-interacting domain. The *BRCA1* mutant Δ500-1863 (Example 1), which lacks both the Rad51-interacting and C-terminal transactivation domains, was used to test whether truncating *BRCA1* mutants retaining the newly-identified p53-interacting domain may act as dominant negative inhibitors of p53-mediated transcription. We predicted that this truncated BRCA1 protein might inhibit p53-mediated transcriptional activity by competing with endogenous BRCA1 for p53. Co-transfection of truncated *BRCA1* (Δ500-1863) with p53 inhibited p53-mediated transcription in a dose-dependent

manner (Fig. 8C). The apparent dominant negative inhibition of p53 was overcome by increasing amounts of exogenous wt BRCA1 (Fig. 8C). It may be that the truncated BRCA1 protein, which does not have a nuclear localization signal, may associate with p53 prior to its entry into the nucleus.

Cooperation between BRCA1 and p53 in apoptosis induction. p53-mediated apoptosis plays a major role in tumor suppression. One mechanism by which p53 mediates apoptosis is by its ability to activate transcription (Haupt et al., 1995, Genes Dev. 9:2170-2183; Chen et al., 1996, Genes Dev. 10:2438-2451). In order to determine if BRCA1 has any effect on p53-mediated apoptosis through its ability to stimulate p53-mediated transcription, p53 was co-transfected with either BRCA1 or its vector into SW480 cells. Apoptotic cells were identified by TUNEL assay and the apoptotic cells (%) was quantified at 40 hrs following transfection (Table 2). Vector-alone transfected cells showed a low level of apoptosis. Transfection of p53 or BRCA1 alone induced apoptosis up to 10-fold and co-transfection of BRCA1 with p53 increased further the apoptotic cells (Table 2) suggesting p53 and BRCA1 may cooperate to induce apoptosis. SW480 cells express low levels of endogenous BRCA1 (Example 1), and so it is possible that the observed levels of apoptosis following p53 transfection alone may already involve cooperation with BRCA1. The modest increase in apoptosis due to exogenous BRCA1 should also be considered in the context that upper limit for (%) apoptosis (range of 20-50%) in these experiments is the transfection efficiency. The results suggest a scenario wherein BRCA1 may enhance the induction of apoptosis by p53 possibly through

stimulation of p53's transcriptional activity.

Table 2.

Plasmids* Used	Apoptotic Cells (%) Expt. #1	Expt. #2	Expt #3
pCEP4+pCR3	2.3	2.1	1.9
pCEP4+*BRCA1*	9.2	7.0	16.6
***p53*+pCR3**	3.4	20.7	11.9
p53*+*BRCA1	11.8	29.1	18.0

*SW480 cells were transfected with *p53* (0.5 μg) or its vector pCEP4 and *BRCA1* (0.5 μg) or its vector pCR3 (0.5 μg) in different combinations. Apoptotic cells were detected by TUNEL assay as described in Materials and Methods. Apoptotic cells show strong fluorescence staining of fragmented chromatin.

C. Discussion

Both p53 and BRCA1 are physically altered by the cellular response to DNA damage, p53 by stabilization and BRCA1 by hyperphosphorylation (Kastan et al., 1991, Cancer Res. 51:6304-6311; Scully et al., 1997, Cell 90:425-435). Both proteins can activate expression of the cell cycle inhibitor *p21* (El-Deiry et al., 1993, *supra*; Example 1). Because both BRCA1 and p53 are implicated in regulation of gene transcription, control of cell growth, and response to DNA damage, we sought to investigate the possibility of a functional cross-talk between these two proteins. The results suggest that BRCA1 and p53 can physically associate, both *in vitro* and *in vivo*, and function in a common pathway of tumor suppression. The ability of BRCA1 to biochemically modulate p53 function suggests that this may be a fundamental role of BRCA1 in tumor suppression. It is

noteworthy that inherited defects of either p53 or BRCA1 can lead to the development of breast cancer in humans (Malkin et al., 1990, Science 250:1233-1238; Miki et al., 1994, Science 266:66-71).

The results reported in this example reveal that BRCA1 is a potent activator of p53-dependent transcription by demonstrating that BRCA1 potentiates p53-activated transcription of the *p21* and the *bax* promoters. The role of p53 as a transcription factor is well established (Vogelstein and Kinzler, 1992, Cell 70:523-526) and appears to be important for its ability to arrest cell cycle progression as well as to induce apoptosis (Levine, 1997, Cell 88:323-331). BRCA1 has been postulated to function as a coactivator of transcription, based on its localization as a component of RNA polymerase II holoenzyme and transactivation function (Example 1 and Chapman and Verma, 1996, Nature 382:678-679; Monteiro et al., 1996, PNAS 93:13595-13599; Scully et al., 1997, PNAS 94:5605-5610). The observation that BRCA1 is a transcriptional coactivator of p53, including strong up-regulation of expression of the *bax* gene, provides a novel mechanism for apoptosis induction and tumor suppression by BRCA1. BRCA1 serves a co-stimulatory role in p53-mediated transcription from *p21*, suggesting BRCA1 is involved in both p53-dependent transcription and p53-independent transcription. This finding is consistent with previous data demonstrating that *p21* can be activated by other p53 independent pathways (El Deiry et al., 1998, Curr. Top. Micro. Immunol. 227:121-137).

During gene expression, a complex of activators, coactivators, and basal factors are necessary to stabilize the interactants and provide transcriptional

specificity. BRCA1 may link a variety of different DNA binding factors such as p53 to the components of the basal transcription machinery. Here we show that p53 and BRCA1 form a specific protein complex *in vitro* and *in vivo*. An N-terminal segment of BRCA1 exon 11 extending from residues 224 to 500 is sufficient for its interaction with p53. Interestingly, exon 11 is deleted in several naturally occurring isoforms of BRCA1 (Thakur et al, 1997, *supra*), leading to speculation that the inability of the BRCA1Δ exon11 mutant to bind to p53 may be a regulatory mechanism for BRCA1. Our finding also suggests that BRCA1 binding to p53 may not be mediated by RAD51, RNA polymerase II holoenzyme or BARD1 since they bind to different regions on BRCA1 (Scully et al, 1997, *supra*; Scully et, al, 1997, Cell 88:265-275; Wu et al, 1996, Nature Gent. 14:430-440). Post-translational modifications of the C-terminus of p53 have been shown to play an import role in controlling p53-specific DNA binding (Levine, 1997, Cell 88:323-331). Phosphorylation, acetylation, antibody binding, or deletion of this region can convert p53 from an inert to an active form for DNA binding (Ko and Prives, 1997, *supra*; Gu and Roeder, 1997, Cell 90:595-606). Therefore allosteric regulation of p53 by distinct cellular signaling pathways modulates the conversion between latent and activated forms of p53. Our results show BRCA1 binds to the C-terminus of p53. BRCA1 may activate p53 by altering the conformation of p53 through direct physical binding and/or modification of the C-terminal p53. In addition, p53 may be bridged to the basal transcriptional machinery by BRCA1.

Mutation in the C-terminus of BRCA1, which contains its transactivation domain (Chapman and Verma,

1996, *supra*; Monteina et al., 199, *supra*), occurs in hereditary breast cancer (Couch and Weber, 1994, Human Mutation 8:8-18). Such mutants of BRCA1 are defective in the ability to activate $p21^{WAF1/CiP1}$ (Example 1). The results reported in this example indicate that the tumor derived BRCA1 mutants carrying point mutations in the C-terminus are also defective in stimulation of p53-dependent transcription. Because BRCA1 interaction with p53 maps to the N-terminal region of BRCA1, we predicted that truncated-BRCA1 proteins, the most common forms found in hereditary breast and ovarian cancer, may compete for sites on p53 and perhaps inhibit p53 function. We found that truncated BRCA1 is a potent dominant negative inhibitor of p53-dependent transcription, and that this could be reversed by excess wild-type BRCA1. These results would predict a defective p53 transcriptional activity in certain cancer cells.

Although p53 and BRCA1 appear to physically interact directly *in vitro*, we found no evidence for stimulation of BRCA1-dependent transactivation of *p21* by exogenous p53 in the absence of p53-DNA binding sites. BRCA1 could, however, greatly stimulate p53-dependent activation of the *bax* promoter, in the absence of p53-independent activation of this promoter by BRCA1. These observations argue that, *in vivo*, certain DNA regulatory elements serve to recruit p53 to certain genes. In such situations the presence of p53 protein favors the chance for local association with BRCA1 and cooperative activation is observed. In our system using either the *p21* or *bax* promoter, the combination of p53 and BRCA1 leads to more than an additive increase in gene expression, but it is not clear whether BRCA1 is required for p53-dependent transcription, because of the lack of

availability of BRCA1-null cell lines. The BRCA1-knockout embryos appear to express *p21* (Hakem et al., 1996, Cell 85:1009-1023) and the current model is that this may be due to genomic instability and subsequent p53 activation (Brugarolas and Jacks, 1997, Nature Med. 7:721-722). The dominant negative action of truncated BRCA1 on p53-dependent transcription hints at the possibility that BRCA1 may be required for p53-dependent transcription. Our results argue that in somatic cells, BRCA1 can activate p53 function leading to tumor suppression.

The induction of apoptosis by p53 is critical for its tumor suppressor function (Symonds et al., 1994, Cell 78:703-711). The results presented in Table 1 suggest that BRCA1 cooperates with p53 in inducing apoptosis. Altogether, our data suggest that BRCA1 acts as a coactivator of p53-driven gene expression and may play an important role in cell growth arrest, apoptosis, and/or DNA damage repair processes. It has not been determined if BRCA1 is required for transcription-independent p53 action. The possibility that the complex of BRCA1 and p53 may directly participate in DNA damage recognition and/or repair could not be excluded. In summary, we have identified a physical and functional association between the tumor suppressors p53 and BRCA1, linking their biochemical effects to a common pathway of tumor suppression.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.

We claim:

1. An isolated nucleic acid molecule comprising a region of an upstream promoter of a *p21* gene, that contains an element required for p53-independent BRCA1-mediated transactivation of the gene.

2. The nucleic acid molecule of claim 1, wherein the region is located on a human *p21* gene between the transcription start site and 143 nucleotides 5' of the transcription start site of the gene.

3. The nucleic acid molecule of claim 2, wherein the region comprises nucleotides 4441-4584 of SEQ ID NO:3.

4. An isolated nucleic acid molecule comprising a region of an upstream promoter of a *p21* gene, the removal of which is associated with prevention of p53-independent transactivation of the gene by BRCA1.

5. The nucleic acid molecule of claim 1, wherein the region is located on a human *p21* gene between 93 and 143 nucleotides 5' of the transcription start site of the gene.

6. The nucleic acid molecule of claim 2, wherein the region comprises nucleotides 4441-4491 of SEQ ID NO:3.

7. An isolated polypeptide comprising a region of a BRCA1 protein that physically interacts with a p53 protein.

8. The polypeptide of claim 7, wherein the region resides within amino acid residues 224-500 of a human BRCA1 protein.

9. The polypeptide of claim 8, wherein the human BRCA1 protein has the amino acid sequence of SEQ ID NO:1.

10. Antibodies immunologically specific for the polypeptide of claim 7.

11. An isolated nucleic acid encoding the polypeptide of claim 7.

12. A vector comprising the nucleic acid of claim 11.

13. An isolated polypeptide comprising a region of a p53 protein that physically interacts with a BRCA1 protein.

14. The polypeptide of claim 13, wherein the region resides within amino acid residues 300-395 of a human p53 protein.

15. The polypeptide of claim 4, wherein the human BRCA1 protein has the amino acid sequence of SEQ ID NO:2.

16. Antibodies immunologically specific for the polypeptide of claim 13.

17. An isolated nucleic acid molecule encoding the polypeptide of claim 13.

18. A vector comprising the nucleic acid molecule of claim 17.

19. An isolated protein-protein complex comprising a p53 protein and a BRCA1 protein.

20. The complex of claim 19, comprising human p53 and human BRCA1.

21. A method for determining if a test compound enhances or interferes with formation of a complex comprising BRCA1 and p53, which comprises:

a) providing an assay test mixture comprising the BRCA1, the p53 and the test compound;

b) providing an equivalent assay control mixture comprising the BRCA1 and the p53, without the test compound;

c) subjecting the test mixture and the control mixture to conditions known to enable formation of the BRCA1-p53 complex in the absence of the test compound;

d) measuring the amount of BRCA1-p53 complexes formed, if any, in the test mixture and the control mixture; and

e) determining if the amount of BRCA1-p53 complex formed in the test mixture is different from the amount of the complex formed in the control mixture, an increase being indicative of the ability of the test compound to enhance formation of the BRCA1-p53 complex and a decrease being indicative of the ability of the test compound to interfere with formation of the BRCA1-p53 complex.

22. The method of claim 21, wherein the assay mixtures are cell-free assay mixtures and the BRCA1 and p53 proteins are provided as isolated proteins.

23. The method of claim 21, wherein the assay mixtures comprise cells, and the BRCA1 and p53 proteins are provided by expression of nucleic acid molecules within the cells.

24. A method for determining if a test compound enhances or interferes with p53-independent BRCA1-mediated transactivation of a *p21* gene, which comprises:

a) providing a cell line deficient in production of endogenous functional BRCA1 and p53, comprising at least one heterologous vector expressing BRCA1, said cell further comprising a reporter gene operably linked to a *p21* promoter containing a region required for the p53-independent BRCA1-mediated transcription, wherein the reporter gene expresses a detectable gene product as a result BRCA1-mediated transactivation of the *p21* promoter;

b) preparing a culture of the cells;

c) incorporating the test compound into the cell culture under conditions permitting expression of the heterologous vectors and the reporter gene, and BRCA1-mediated transactivation of the *p21* promoter; and

d) measuring expression of the reporter gene by detecting the presence or amount, if any, of the detectable gene product and comparing the expression to an equivalent cell culture in which the test compound was not incorporated, an increase in expression of the reporter gene in the culture containing the test compound

being indicative that the test compound enhances p53-independent BRCA1-mediated transactivation of the *p21* gene, a decrease in expression of the reporter gene being indicative that the test compound interferes with p53-independent BRCA1-mediated transactivation of the *p21* gene.

25. The method of claim 24, wherein the cells lack endogenous functional proteins selected from the group consisting of BRCA1 and p53.

26. A method for determining if a test compound enhances or interferes with tumor suppression resulting from p53-independent BRCA1-mediated transactivation of a *p21* gene, which comprises:

a) providing a tumor cell line comprising at least one heterologous vector expressing BRCA1;

b) preparing a culture of the cells;

c) incorporating the test compound into the cell culture under conditions permitting expression of the heterologous vector and BRCA1-mediated transactivation of an endogenous *p21* gene; and

d) determining proliferation of the cell culture and comparing it to proliferation of an equivalent cell culture in which the test compound was not incorporated, an increase in the proliferation of the culture containing the test compound being indicative that the test compound interferes with tumor suppression, a decrease in the proliferation of the culture containing the test compound being indicative that the test compound enhances tumor suppression.

27. The method of claim 26, wherein the cells lack endogenous functional proteins selected from the group consisting of BRCA1 and p53.

28. The method of claim 26, wherein the proliferation rate is measured by measuring DNA synthesis in the cultured cells.

29. A method for determining if a test compound enhances or interferes with p53-dependent BRCA1-mediated transactivation of a gene, which comprises:

a) providing a cell line comprising at least one heterologous vector expressing BRCA1 and p53, said cell further comprising a reporter gene operably linked to a promoter of a gene comprising sequences enabling p53-dependent BRCA1-mediated transactivation of the gene, wherein the reporter gene expresses a detectable gene product as a result BRCA1-mediated transactivation of the *p21* promoter;

b) preparing a culture of the cells;

c) incorporating the test compound into the cell culture under conditions permitting expression of the heterologous vectors, reporter gene and p53-dependent BRCA1-mediated transactivation of the promoter; and

d) measuring expression of the reporter gene by detecting the presence or amount, if any, of the detectable gene product and comparing the expression to an equivalent cell culture in which the test compound was not incorporated, an increase in expression of the reporter gene in the culture containing the test compound being indicative that the test compound enhances p53-dependent BRCA1-mediated transactivation of the gene, a decrease in expression of the reporter gene being indicative that the test compound interferes with p53-dependent BRCA1-mediated transactivation of the gene.

30. The method of claim 29, wherein the cells lack endogenous functional proteins selected from the group consisting of BRCA1 and p53.

31. A method for determining if a test compound enhances or interferes with tumor suppression resulting from p53-dependent BRCA1-mediated transactivation of a tumor suppressor gene, which comprises:

a) providing a tumor cell line comprising at least one heterologous vector expressing BRCA1 and p53;

b) preparing a culture of the cells;

c) incorporating the test compound into the cell culture under conditions permitting expression of the heterologous vectors and p53-dependent BRCA1-mediated transactivation of the gene; and

d) determining proliferation of the cell culture and comparing it to proliferation of an equivalent cell culture in which the test compound was not incorporated, an increase in the proliferation of the culture containing the test compound being indicative that the test compound interferes with tumor suppression, a decrease in the proliferation of the culture containing the test compound being indicative that the test compound enhances tumor suppression.

32. The method of claim 31, wherein the cells lack endogenous functional proteins selected from the group consisting of BRCA1 and p53.

33. The method of claim 31, wherein the gene is selected from the group consisting of *bax* and *p21*.

34. The method of claim 31, wherein the proliferation is determined by measuring DNA synthesis in the cultured cells.

35. The method of claim 31, wherein the proliferation is determined by measuring apoptosis in the cultured cells.

36. A kit for performing the method of claim 21, comprising a container containing at least one biological molecule selected from the group consisting of BRCA1 protein, p53 protein, an expressible nucleic acid molecule encoding BRCA1, an expressible nucleic acid molecule encoding p53, and a reporter gene.

37. A kit for performing the method of claim 24, comprising a container containing at least one biological molecule selected from the group consisting of an expressible vector encoding BRCA1, and the reporter gene.

38. The kit of claim 37, which further comprises the tumor cell line.

39. A kit for performing the method of claim 26, which comprises a container containing an expressible vector encoding BRCA1.

40. The kit of claim 39, which further comprises the tumor cell line.

41. A kit for performing the method of claim 29, comprising a container containing at least one

biological molecule selected from the group consisting of an expressible vector encoding BRCA1, an expressible vector encoding p53, and the reporter gene.

42. The kit of claim 41, which further comprises the tumor cell line.

43. A kit for performing the method of claim 31, which comprises a container containing an expressible vector encoding BRCA1 and an expressible vector encoding p53.

44. The kit of claim 43, which further comprises the tumor cell line.

45. A method for diagnosing a subject's predisposition for developing a tumor, comprising detecting defects in the subject's *BRCA1* genes, BRCA1 proteins or *p21* promoters that result in reduction or prevention of p53-independent BRCA1-mediated transactivation of the subject's *p21* genes.

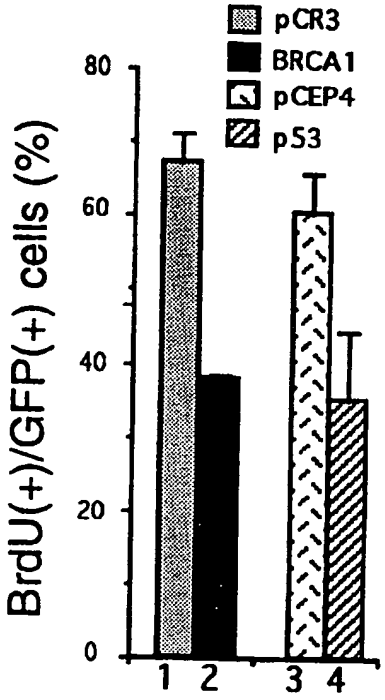
46. A method for diagnosing a subject's predisposition for developing a tumor, comprising detecting defects in the subject's *BRCA1* genes, BRCA1 proteins, *p53* genes, p53 proteins, or promoters of tumor suppressor genes co-activated by BRCA1 and p53, that result in reduction or prevention of p53-dependent BRCA1-mediated transactivation of the tumor suppressor genes.

47. A method for enhancing tumor suppression which comprises increasing p53-dependent or -independent BRCA1-mediated transactivation of at least one tumor

suppressor gene in tumor cells.

48. A method for increasing proliferation of cells, which comprises decreasing p53-dependent or -independent BRCA1-mediated transactivation of at least one tumor suppressor gene in the cells.

Fig. 1



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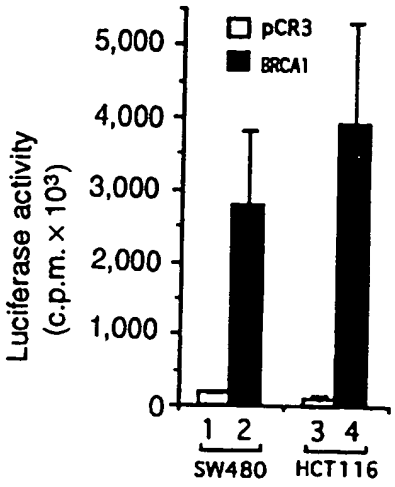

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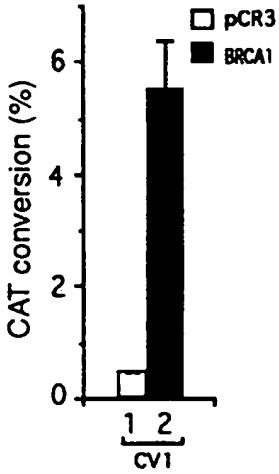
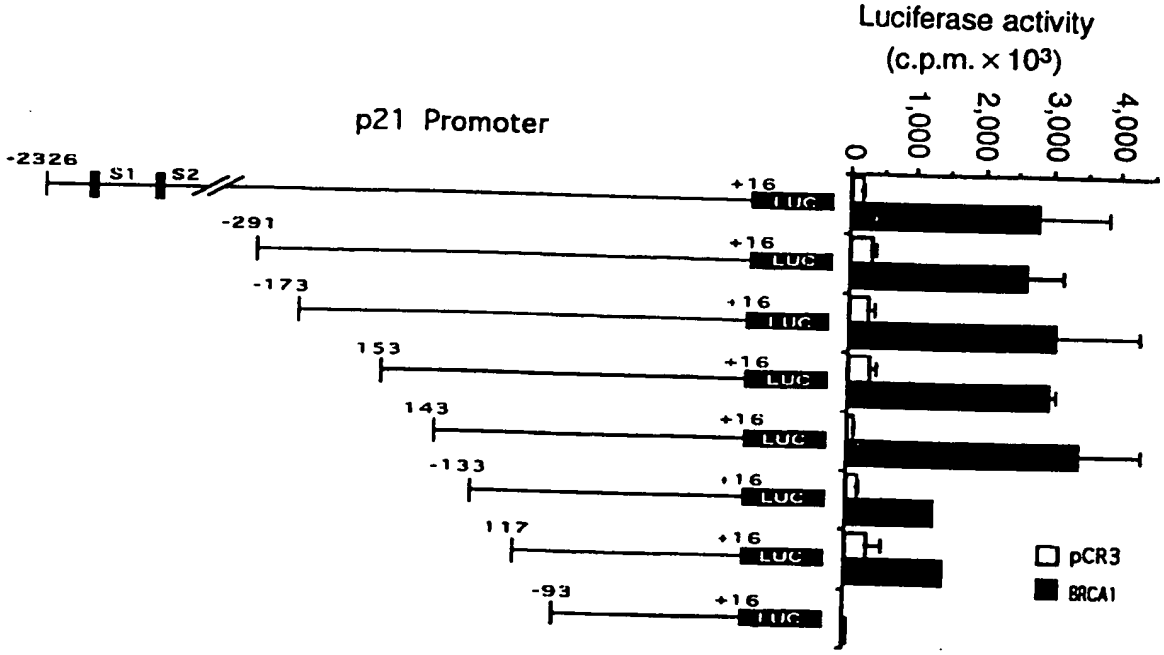


Fig. 2C



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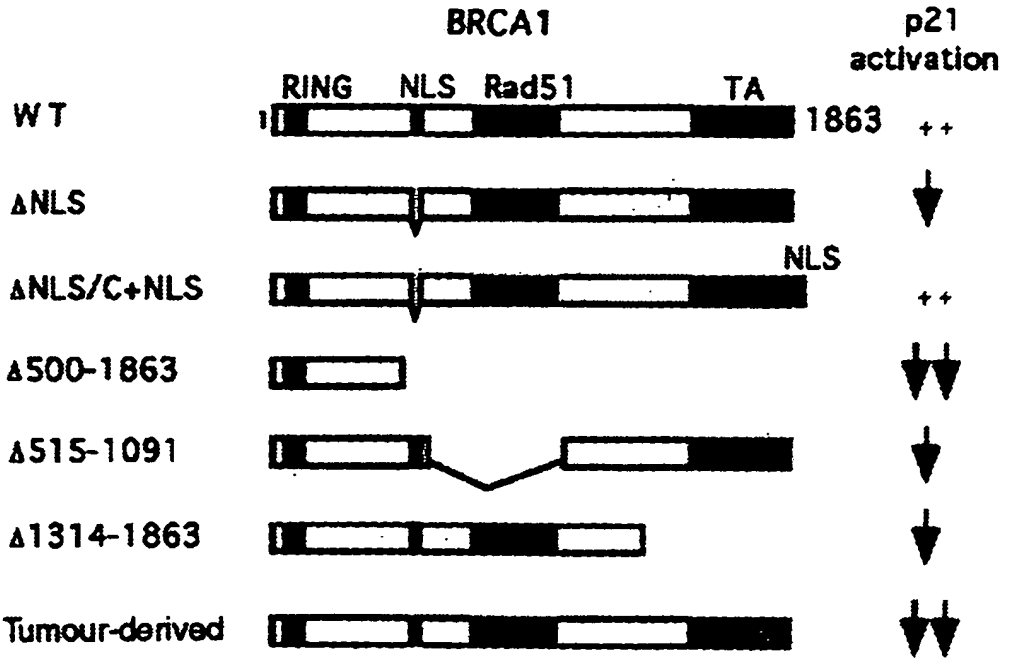

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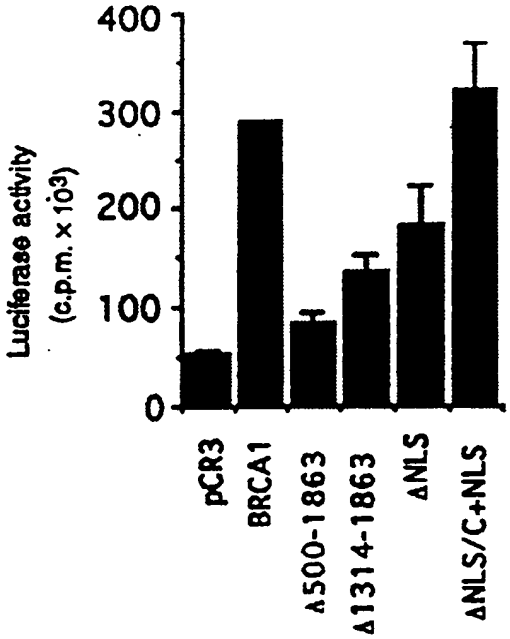

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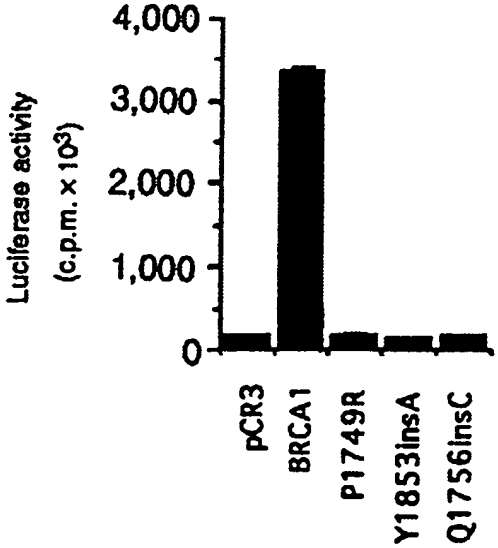

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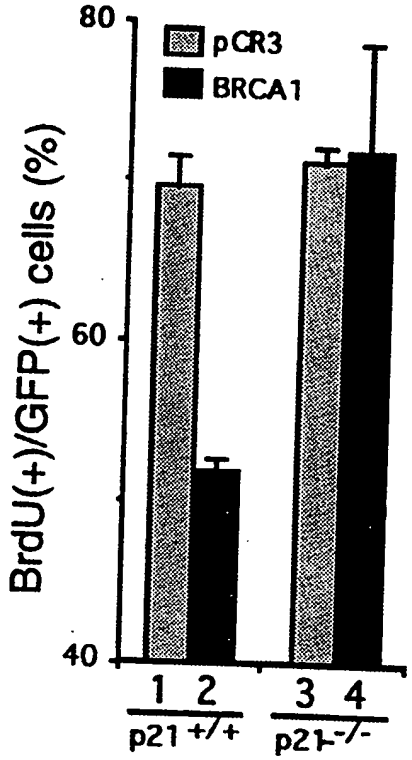

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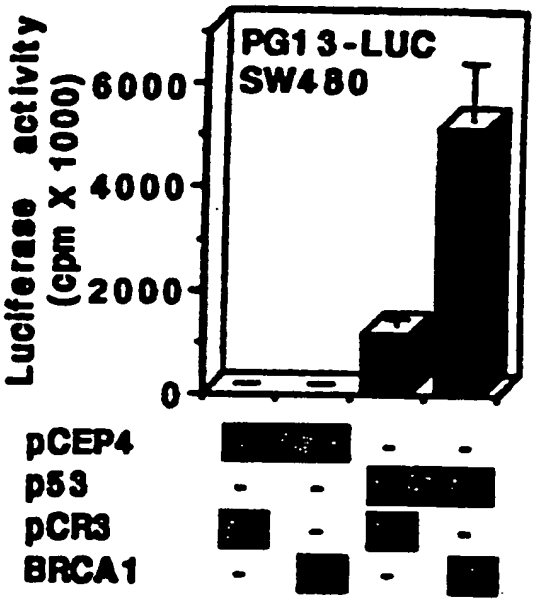

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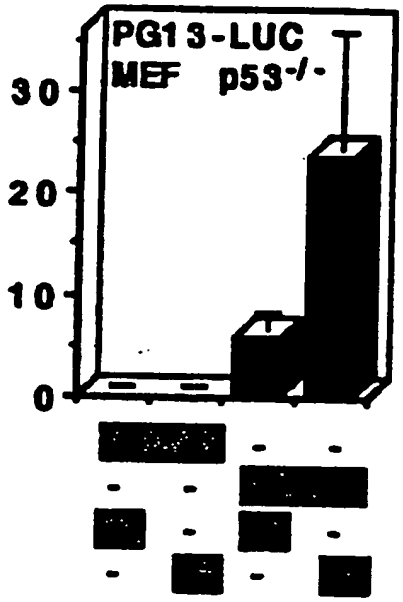

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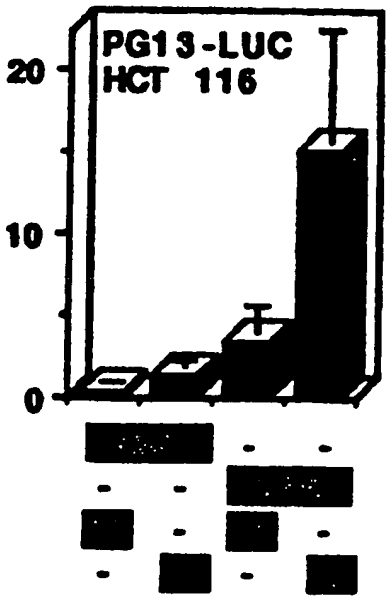

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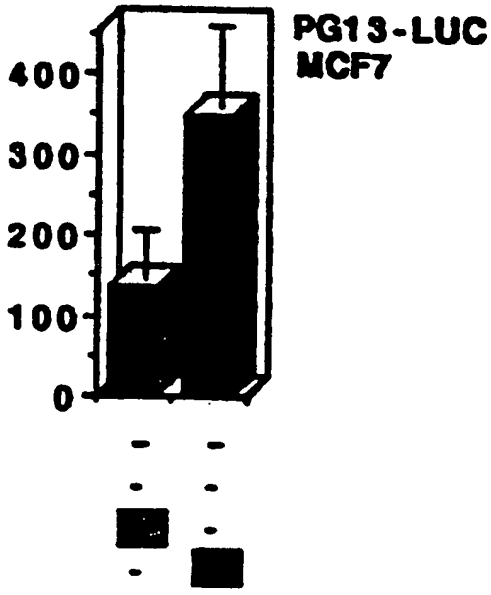

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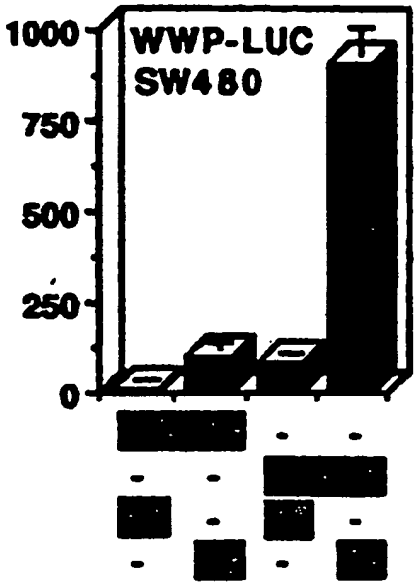

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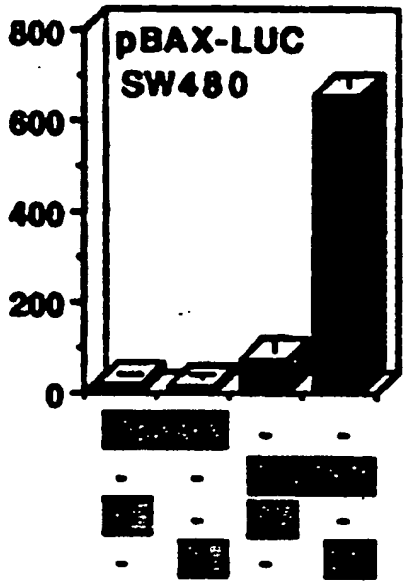

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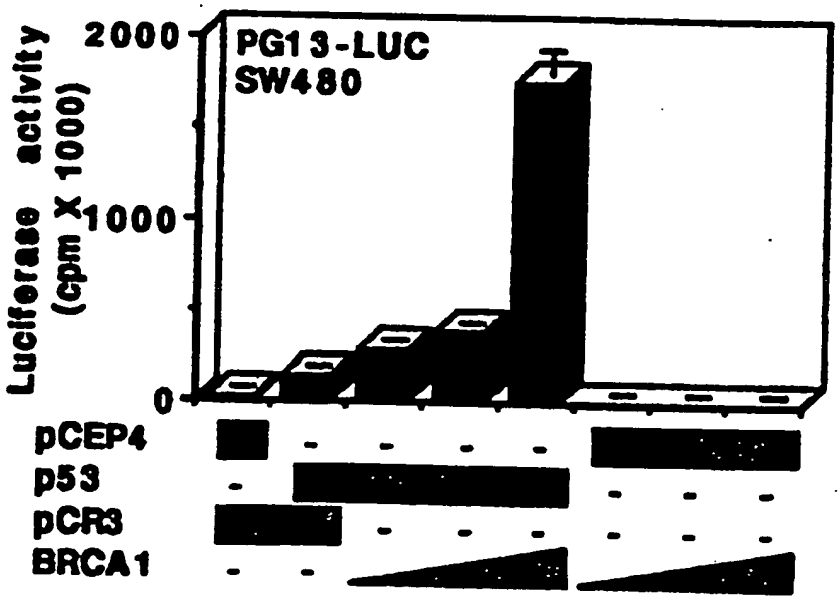


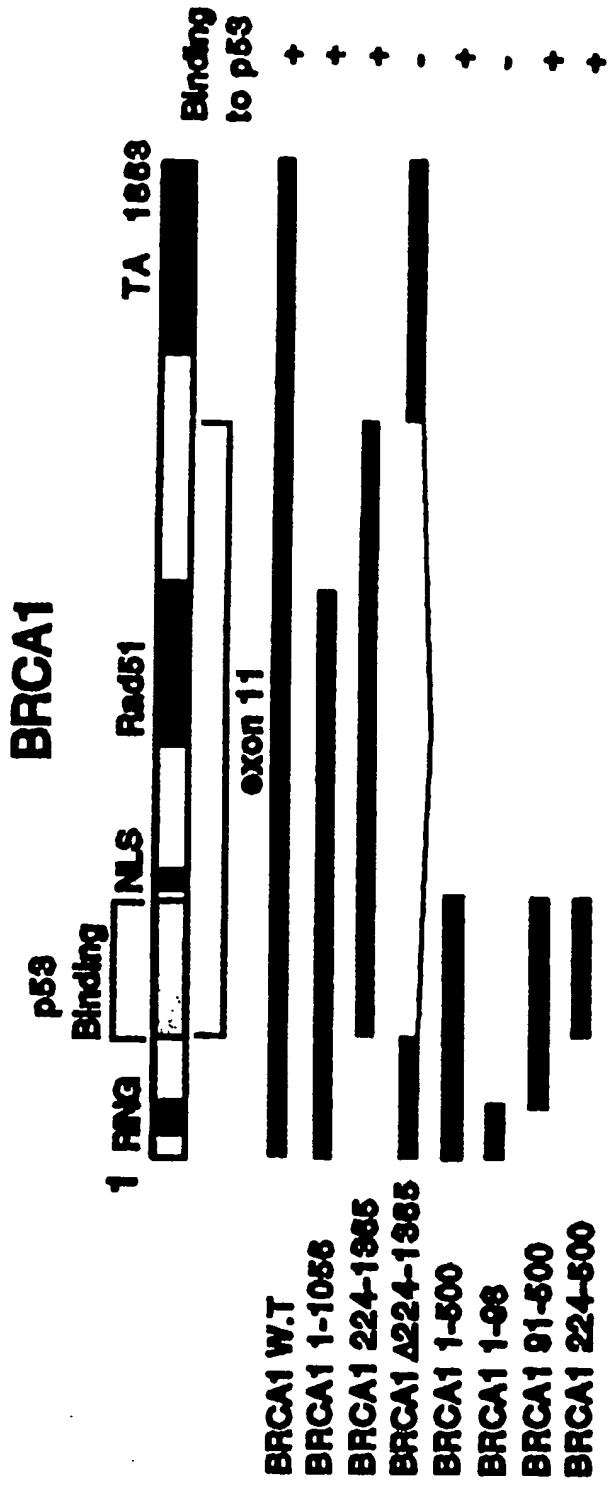


Fig. 6

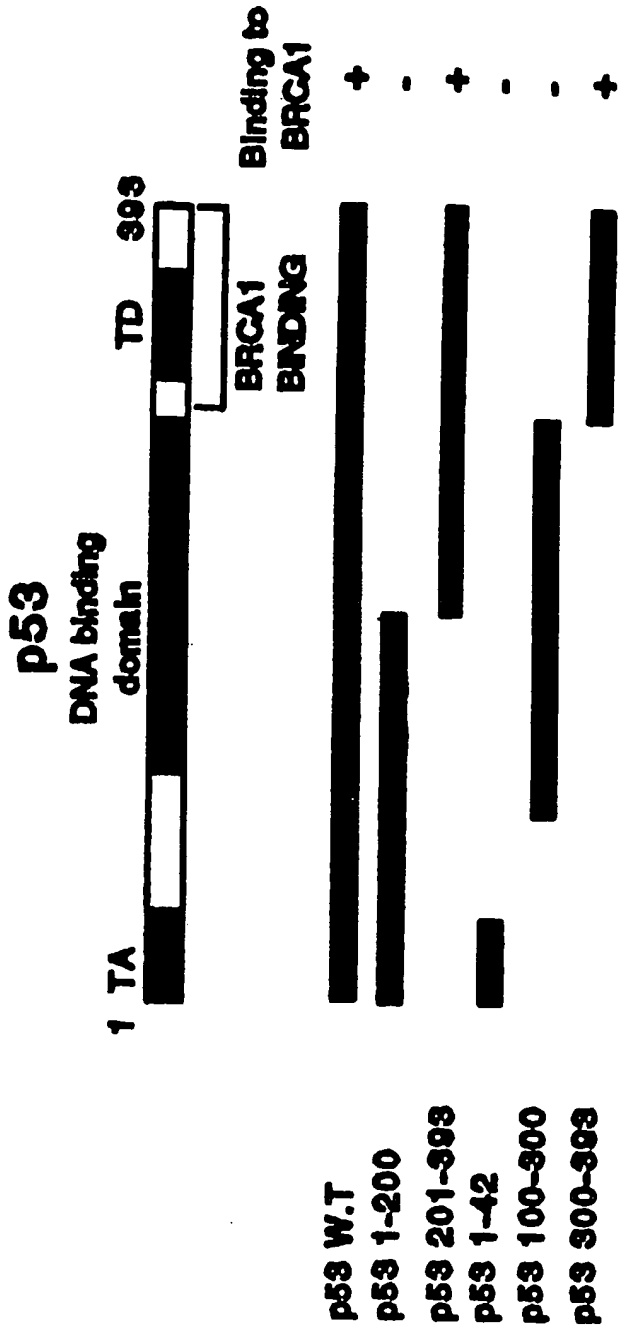
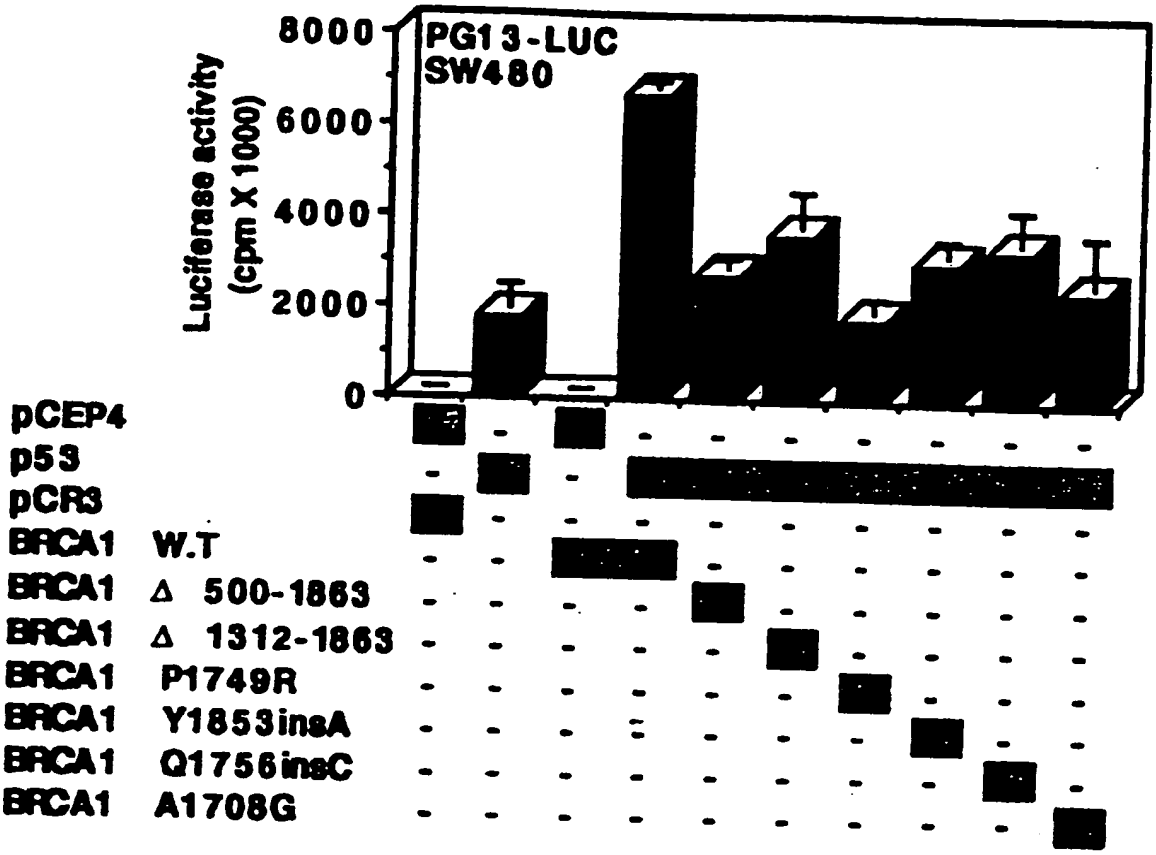


Fig. 7

Fig. 8A



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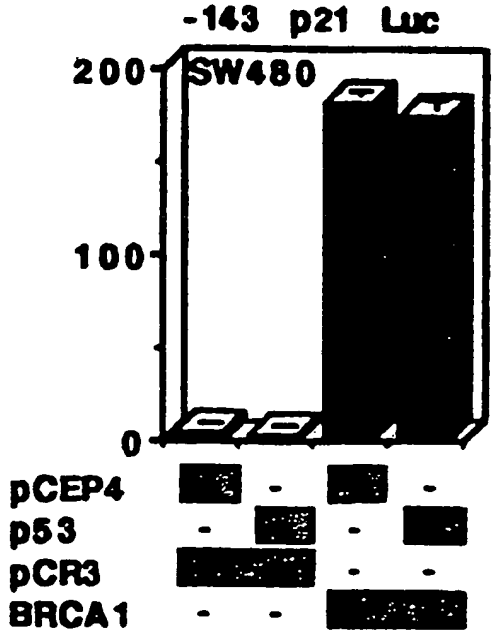

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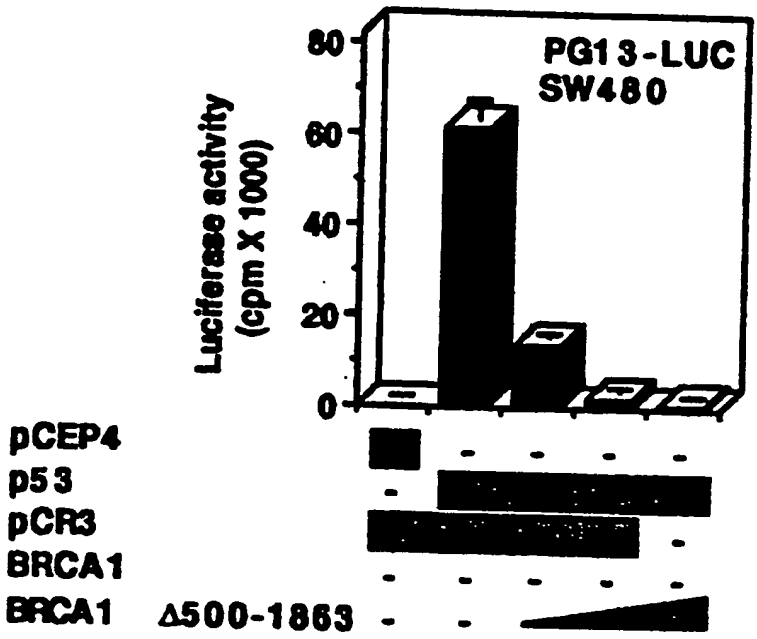

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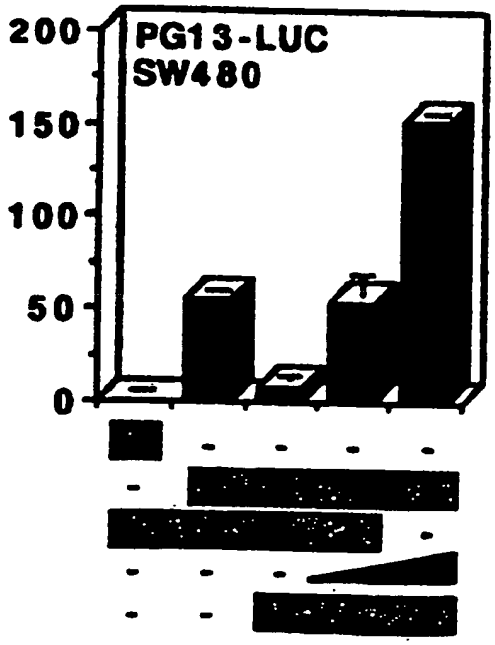

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07150

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 1/00; C07H 21/02
US CL : 530/350; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Genbank, SEQ ID NO:1 and 3, p21 gene, isolated, promoter, upstream p53

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENBANK Sequence Database, National Center for Biotechnology Information, National Library of Medicine, (Bethesda, Maryland, USA) Accession U24170, El-Deiry et al., 'Topological Control of P21 WAF1/CIP1 Expression in Normal and Neoplastic Tissues,' abstract, Cancer Res. 55 (13), 2910-2919, 20 July 1995, see entire sequence listing.	1-6
X	EL-DEIRY et al. Topological Control of P21 WAF1/CIP1 Expression in Normal and Neoplastic Tissues. Cancer Research. July 1995, Vol. 55, pages 2910-2919, see entire article.	1-6
X	MIKI et al. A Strong Candidate for the Breast and Ovarian Cancer Susceptibility Gene BRCA1. Science. 07 October 1994, Vol. 266, pages 66-71, see especially Figure 2, page 67.	7-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
21 JUNE 1999	28 JUL 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SUSAN UNGAR
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07150

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-9

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-6, drawn to an isolated nucleic acid molecule comprising a region of an upstream promotor of a p21 gene which contains an element required for p53-independent BRCAI-mediated transactivation of the gene.
Group II, claim(s) 7-9, drawn to an isolated polypeptide comprising a region of BRCAI protein.
Group III, claim(s) 10, drawn to an antibody specific for BRCAI protein.
Group IV, claim(s) 11-12, drawn to an isolated nucleic acid encoding BRCAI protein and a vector comprising said nucleic acid.
Group V, claim(s) 13-14, drawn to an isolated polypeptide comprising a region of p53.
Group VI, claim(s) 16, drawn to an antibody specific for p53.
Group VII, claim(s)17-18, drawn to a nucleic acid molecule encoding p53 and a vector comprising said nucleic acid.
Group VIII, claim(s) 19-20, drawn to an isolated protein-protein complex comprising p53 and BRCA1.
Group IX, claim(s) 21-23 and 36, drawn to a method of determining if a test compound modulates the formation of a complex comprising BRCA1 and p53.
Group X, claim(s)24, 25, 37 and 38, drawn to a method for determining if a test compound enhances or interferes with p53-independent BRCAI-mediated transactivation of a p21 gene.
Group XI, claim(s) 26-28 and 39-40, drawn to a method for determining if a test compound modulates tumor suppression resulting from p53-independent BRCA1-mediated transactivation of a p21 gene.
Group XII, claim(s) 29-30 and 41-42, drawn to a method for determining if a test compound modulates p53-independent BRCA1-mediated transactivation of a gene.
Group XIII, claim(s)31-35 and 43-44, drawn to a method for determining if a test compound modulates tumor suppression resulting from p53-dependent BRCA1-mediated transactivation of a tumor suppressor gene.
Group XIV, claim(s) 45-46, drawn to a method for diagnosing predisposition for developing a tumor.
Group XV, claim(s) 47, drawn to a method for enhancing tumor suppression.
Group XVI, claim 48, drawn to a method for increasing cell proliferation.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

Group XIV
Species A, Claims 45 and 46, a method for diagnosing predisposition for developing a tumor comprising detecting defects in the subjects BRCA1 genes.

Species B, Claims 45 and 46, a method for diagnosing predisposition for developing a tumor comprising detecting defects in the subjects BRCA1 proteins.

Species C, Claims 45 and 46, a method for diagnosing predisposition for developing a tumor comprising detecting defects in the subjects p21 promoters.

Species D, Claim 46, a method for diagnosing predisposition for developing a tumor comprising detecting defects in the subjects p53 genes.

Species E, Claim 46, a method for diagnosing predisposition for developing a tumor comprising detecting defects in the subjects p53 proteins.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07150

The inventions listed as Groups I-XVI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking groups I-XVI appears to be an isolated nucleic acid molecule comprising a region of an upstream promoter of a p21 gene that contains an element required for p53-independent BRCA1-mediated transactivation of the gene, wherein the region comprises nucleotides 4441-4591 of SEQ ID NO:3.

However, El-Deiry et al(HSU24170), Genbank Sequence Database (Accession U24170), National Center for Biotechnology Information, National Library of Medicine, Bethesda, Maryland, publicly available 20 July 1995 teaches an isolated nucleic acid molecule which has 100% identity to SEQ ID NO:3.

Therefore, the technical feature linking Groups I-XVI is not considered to be a special technical feature as defined by PCT Rule 13.2 as it does not define a contribution over the prior art.

The special technical feature of Group I is considered to be an isolated nucleic acid molecule comprising a p53-independent p21 promoter.

The special technical feature of Group II is considered to be a BRAC1 protein.

The special technical feature of Group III is considered to be an antibody specific for BRAC1.

The special technical feature of Group IV is considered to be a nucleic acid encoding BRAC1.

The special technical feature of Group V is considered to be a p53 protein.

The special technical feature of Group VI is considered to be an antibody specific for p53.

The special technical feature of Group VII is considered to be a nucleic acid encoding p53.

The special technical feature of Group VIII is considered to be a protein complex.

The special technical feature of Group IX is considered to be a method of determining if a test compound modulates formation of a BRCA1/p53 complex.

The special technical feature of Group X is considered to be a method for determining if a test compound modulates p53-independent BRCA1-mediated transactivation of a p21 gene.

The special technical feature of Group XI is considered to be a method for determining if a test compound modulates p53-independent BRCA-1 mediated transactivation of a p21 gene.

The special technical feature of Group XII is considered to be a
method for determining if a test compound modulates p53-independent BRCA-1 mediated transactivation of a p21 gene.

The special technical feature of Group XIII is considered to be a method for determining if a test compound modulates tumor suppression.

The special technical feature of Group XIV is considered to be a method for diagnosis of predisposition for developing a tumor.

The special technical feature of Group XV is considered to be a method for enhancing tumor suppression.

The special technical feature of Group XVI is considered to be a method for increasing cell proliferation.

Accordingly, Groups I-XVI are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

The species as claimed are materially distinct methods which differ at least in objectives, method steps, reagents and/or dosages and/or schedules used, response variables, and criteria for success.